Functional characterisation of human CD4+CD25+ regulatory T cells and the transcriptional regulator, FOXP3

Thesis

UNRESTRICTED

Functional Characterisation of Human CD4+CD25+ Regulatory T cells and the Transcriptional Regulator, FOXP3.

Emma Louise Smith BSc (Hons)

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

June 2005

Department of Cell Biology

UCB-Celltech, 216 Bath Road, Slough,

Berkshire SL1-4EN, U.K.

DATE OF SUBMISSION 02 JUNE 2005
DATE OF AWARD 22 AUGUST 2005

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Naturally occurring CD4+CD25+ regulatory T cells are thought to play an essential role in the maintenance of immunological self-tolerance by actively suppressing auto-reactive T lymphocytes. Exploitation of these cells could provide novel and effective therapies for the treatment of various clinical diseases, including autoimmune diseases and cancer. However the biology surrounding these cells is complex and there are many unresolved issues, including a lack of specific markers for these cells and an incomplete understanding of the mechanism of action of these cells.

The initial experiments described in this thesis attempted to identify cell surface markers that were specific to the human CD4+CD25+ regulatory T cell population. Functional assays were conducted in parallel in an attempt to identify cell surface molecules and cytokines that might be responsible for the cell-mediated suppression. The lack of identification of a specific molecule responsible for the suppressor phenotype highlights the complexity of this cell population.

As these studies progressed, a new marker of CD4+CD25+ regulatory T cells was reported called FOXP3. Subsequent experiments described in these studies focussed on investigating the effects of overexpressing human FOXP3 in human T cells. A novel cotransfection system was devised whereby the gene encoding *FOXP3* was cotransfected with DNA encoding a specific receptor involved in T-cell activation (chimeric receptor). The data generated showed that overexpression of FOXP3 in resting human CD4+ and CD8+ T cells resulted in a significant inhibition of subsequent T cell activation. Splice variant forms of the FOXP3 protein were also investigated and were shown to possess potent repressor activity.

Acknowledgements

Firstly I would like to thank my supervisors, Dr. Martyn Robinson and Dr. Andrew Nesbitt for their support, encouragement and enthusiasm throughout these studies. Both of you always found time for me, even when there were more important issues to deal with and for that I am extremely grateful. I must also thank my external supervisor, Dr. Fiona Powrie for her scientific advice and helpful discussions. I would also like to thank Dr. Tim Bourne for encouraging me to think more deeply about my results and also for all the time he devoted to reading the thesis. Hopefully I have managed to address most of your comments. I must also thank Dr. Fred Ramsdell and Dr. Roli Khattri for their support and enthusiasm for the FOXP3 studies.

Many colleagues have also shared their time and technical expertise to help the practical side of the work progress. Special thanks go to Dr. Patrick Slocombe for all the help with cloning. Thanks also to Dr. Gill Holdsworth for her Western blotting skills, Dr. Luca Fossati for help with the microscopy and Dr. Stephen Rapecki for the Luminex analyses. I am also incredibly grateful to Dr. Helene Finney for all the help and advice with the chimeric receptor co-transfection system. I'd also like to thank all my colleagues in L235 and L242a, for their support and helpful discussions. Special thanks go to Marianne Bergin and Dr. Lisa Skelton for their continued support throughout these studies.

I would also like to thank my family for all their support, I'm sorry if I've often seemed preoccupied with other things. Finally, I want to thank Steve for his total commitment and belief in me. These last three years have been tough but you have always been there for me, both emotionally and practically. Here's to the future!

Abbreviations

A	ampere
A (aa)	alanine
A (nt)	adenine
aa	amino acid
AICD	activation induced cell death
AIRE	autoimmune regulator
Amp	ampicillin
AMPS II	autoimmune polyglandular syndrome II
APC	antigen presenting cell
ANOVA	analysis of variance
BGH	bovine growth hormone
bp	base pair
B. pertussis	*Bordetella pertussis*
BDCA	blood dendritic cell antigen
BSA	bovine serum albumin
C	Celsius
C (aa)	cysteine
C (nt)	cytosine
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFA	complete Freund's adjuvant
CHO	Chinese hamster ovary
Ci	Curie
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CR	chimeric receptor
CsA	cyclosporin A
CTLA-4	cytotoxic T lymphocyte-associated molecule-4
D (aa)	aspartic acid
Da	Dalton
DC	dendritic cell

dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DP	double positive
E (aa)	glutamic acid
ECACC	European Collection of Animal Cell Cultures
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
F (aa)	phenylalanine
Fab'	fragment, antigen binding
FACS	fluorescence activated cell sorter
Fc	fragment crystallisable
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FKH	forkhead
FOXP3	forkhead box protein 3 (mouse and human protein)
Foxp3	forkhead box protein 3 (mouse gene)
FOXP3	forkhead box protein 3 (human gene)
FSC	forward scatter
FWD	forward
g	acceleration relative to that due to the Earth's gravitational field
G (aa)	glutamic acid
G (nt)	guanine
G1	extracellular spacer region comprising human IgG1 hinge, CH2, CH3
GC	glucocorticoid
GFP	green fluorescent protein
GITR	glucocorticoid-induced tumour necrosis receptor
GM-CSF	granulocyte macrophage-colony stimulating factor
H (aa)	histidine
HA	haemagglutinin

H. hepaticus	*Helicobacter hepaticus*
HEL	hen egg lysosyme
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HO-1	heme oxygenase-1
HRP	horseradish peroxidase
hu	human
I (aa)	isoleucine
IAA	isoamyl alcohol
IBD	inflammatory bowel disease
ICOS	inducible costimulator
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon-gamma
Ig	immunoglobulin
IL-	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	immunoreceptor tyrosine activation motif
K (aa)	lysine
kb	kilobase
kDa	kilodalton
KLH	keyhole limpet haemocyanin
L	litre
L (aa)	leucine
LAG-3	lymphocyte activation gene-3
LB medium	Luria-Bertani medium
LCMV	lymphocytic choriomenigitis virus
L. major	*Leishmania major*
LFA-1	lymphocyte function associated antigen-1
LPS	lipopolysaccharide
LT	lymphotoxin
m	milli
M	molar

M (aa)	methionine
mAb	monoclonal antibody
MACS	magnetic cell sorting
MBP	myelin basic protein
MHC	major histocompatability complex
MOG	myelin oligodendrocyte glycoprotein
MP	matrix peptide
MS	multiple sclerosis
mu	mouse
n	nano
N (aa)	asparagine
N (nt)	adenine, cytosine, guanine, thymine
NEAA	non-essential amino acids
NFAT	nuclear factor activated T cells
NF-κB	nuclear factor κB
NK	natural killer
nt	nucleotide
ova	ovalbulmin
P (aa)	proline
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
PE	phycoerythrin
PHA-L	*Phaseolus vulgaris* leucoagglutinin
PI	propidium iodide
PI3-K	phosphatidylinositol 3-kinase
PLA_2	phospholipase A_2
PMA	phorbol-12-myristate, 13-acetate
PV	Pichinde virus
PVDF	polyvinylidene difluoride
Q (aa)	glutamine

R	receptor
R (aa)	arginine
RA	rheumatoid arthritis
RAG	recombinase activator gene
REV	reverse
RPMI	Rosweli Park Memorial Institute
RNA	ribonucleic acid
S (aa)	serine
SCID	severe combined immunodeficiency
scFv	single chain fragment of antibody variable regions
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	short interfering RNA
SLE	systemic lupus erythematosus
SSC	side scatter
SV40	Simian virus 40
T (aa)	threonine
T (nt)	thymine
TAE	tris-acetate EDTA
TCR	T cell receptor
TE	tris EDTA
TGFβ	transforming growth factor-beta
Th1	T helper 1 T cells
Th2	T helper 2 T cells
Th3	T helper 3 T cells
TLR	toll like receptor
TM	transmembrane
TNE	high salt tris EDTA
TNFα	tumour necrosis factor-alpha
TNFR	tumour necrosis factor receptor
Tr1	T regulatory type 1 T cells
TRAF	TNF receptor-associated factor

Tris	trizma base
TT	tetanus toxoid
U	units
UTR	untranslated region
uv	ultraviolet
V (aa)	valine
v/v	volume/volume
W (aa)	tryptophan
w/v	weight/volume
Y (aa)	tyrosine
µ	micro

Contents

List of Figures

List of Tables

Chapter One

Introduction

1.1 The immune system

The function of the mammalian immune system is to protect the host from invading pathogens. The cellular and molecular mechanisms that regulate this system although highly complex, generate a dynamic and extremely efficient network of protective responses. The ability of the immune system to recognise and destroy invading pathogens requires a high degree of receptor diversity and consequently the immune system has evolved additional mechanisms to distinguish between self and non-self antigens. This feature of the immune system, termed immune tolerance, is crucial to prevent the development of destructive immunopathologies against self antigen.

1.2 Thymic selection

Negative selection of self-reactive T cells in the thymus is an important process contributing to immune tolerance. Lymphoid progenitor cells arrive from the bone marrow and seed the thymus. The thymus contains two distinct anatomical areas, an outer region called the cortex and an inner region called the medulla. Within the cortex there are a small population of cluster of differentiation (CD) 4 and CD8- double negative thymocytes. These cells undergo a proliferative expansion and differentiation step which is driven by the pre-T cell receptor, resulting in the generation of CD4+CD8+ double positive (DP) thymocytes, each expressing a clonotypic αβ T cell receptor (TCR). The DP thymocytes interact with major histocompatability complex (MHC)-self peptide complexes displayed by cortical epithelial cells. At this stage the majority of the DP thymocytes (approximately 90%) die 'by neglect' due to a failure of the TCRαβ to engage with a peptide-MHC ligand.

Positive selection of thymocytes occurs when the TCR of the thymocyte engages a peptide-MHC ligand with low affinity and receives a low-level TCR signal that induces survival and differentiation into single positive CD4+CD8- and CD4-CD8+ thymocytes. These cells are then exported from the thymus and populate the peripheral lymphoid organs. Approximately 5% of thymocytes are believed to undergo positive selection (Palmer, 2003; Sprent and Kishimoto, 2002)

Negative selection occurs when the TCR of a thymocyte engages with a peptide-MHC ligand with high affinity. This strong signal through the TCR induces apoptosis of the thymocyte. The location of negative selection within the thymus is an area of controversy. Some studies suggest that both positive and negative selection occur in the cortex. However negative selection is thought to require costimulatory signals as well as signalling through the TCR. The cortical epithelial cells lack the expression of costimulatory molecules such as CD80 and CD86, suggesting these cells are inefficient at inducing apoptosis. Conversely, the medulla region of the thymus is packed with antigen presenting cells (APCs), consisting of both dendritic cells and medullary epithelial cells that do express these molecules. The medulla is also readily accessible to soluble antigens entering the thymus from the bloodstream. Medullary epithelial cells have also been shown to be sites of promiscuous gene expression and to transcribe genes that are normally expressed in peripheral tissues (Palmer, 2003). Autoimmune regulator (AIRE) has recently been identified as a transcription factor expressed by medullary epithelial cells that promotes the expression of tissue-specific antigens, suggesting that AIRE is critical for thymic negative selection (Anderson *et al.*, 2002). Indeed loss-of-function mutations in this gene can lead to autoimmune

disease, highlighting the importance of negative selection in the generation of immune tolerance (Pitkanen and Peterson, 2003; Ramsdell and Ziegler, 2003).

Whilst negative selection plays an important role in generating central immune tolerance, autoimmune diseases do occur and there is evidence for the presence of autoreactive T cells in normal healthy individuals (Danke *et al.*, 2004). This suggests that the process of elimination of self-reactive T cells within the thymus is not infallible and may be because certain antigens are not expressed within the thymus, or that they are not expressed at high enough levels to induce tolerance.

1.3 TCR cross-reactivity

Whilst it once appeared that each TCR was specific for only one peptide (the 'one clonotype-one specificity' paradigm (Mason, 1998)), this is now believed not to be true. Recent studies suggest that the TCR is highly cross-reactive and it is postulated that a single TCR may recognise in the region of 10^6 different peptide ligands (Mason, 1998). This apparent high degree of cross-reactivity can be beneficial to the host organism by providing increased protective immunity. Studies in the mouse have shown that prior infection with lymphocytic choriomeningitis virus (LCMV) provided partial protection against Pichinde virus (PV). Although these viruses show only limited sequence similarities, CD8+ T cells isolated from mice infected with either virus, were shown to cross-react with a nucleoprotein-derived peptide from the other virus (Brehm *et al.*, 2002).

Whilst TCR cross-reactivity ensures that a sufficient number of T cells recognise novel pathogens and mount an effective immune response, the trade off is an increased potential for T cells to see self-antigens. Indeed studies with human myelin basic protein (MBP)-specific T cell clones, isolated from the blood of multiple sclerosis (MS) patients, have demonstrated that these cells can be activated by various microbial peptides (Wucherpfennig and Strominger, 1995). These data suggest that T cells reactive with both self and foreign peptides are present in the T cell repertoire. In addition, autoimmune diseases can be induced in animal models following immunisation with self-antigen in adjuvant (Sakaguchi, 2000). However, despite this high degree of cross-reactivity, the incidence of autoimmunity is low. This suggests that there must be other mechanisms operating to maintain peripheral tolerance to self.

1.4 Peripheral mechanisms of tolerance

A number of different mechanisms, dependent upon the interaction of the TCR with MHC-peptide complexes, have been proposed to account for the maintenance of peripheral self-tolerance. During an immune response to an invading pathogen, a fully mature APC displaying antigen and expressing various costimulatory molecules, interacts with a naïve T cell resulting in the generation of fully activated T cells that exhibit potent effector functions. However, in the absence of inflammation or infection, presentation of antigen by immature APCs will result in T cell unresponsiveness or anergy. Thus the presentation of self-antigens in the periphery in the absence of danger signals is believed to be one of the mechanisms of peripheral tolerance (Appleman and Boussiotis, 2003; Matzinger, 2002). Other suggested

mechanisms for peripheral self-tolerance include T cell deletion via activation induced T cell death (Miller and Basten, 1996) and immunological ignorance, which is thought to occur when self-antigens are expressed at low levels (Zinkernagel *et al.*, 1997).

Whilst these mechanisms certainly play a role in controlling T cell responses in the periphery, they do not account for the observation that in numerous experimental animal models of autoimmune disease, onset of disease can be prevented following the reconstitution of the animals with certain T cell populations (Shevach, 2000). These data lead to the conclusion that there must be a specialised subset of T cells which have the ability to contribute to T cell tolerance in the periphery.

1.5 The Regulatory T cell

The concept of a specialised subset of T cells with an inherent ability to suppress immune responses was first proposed in 1970 (Gershon and Kondo, 1970). However the cellular and molecular mechanisms responsible for the mode of action of these cells, termed suppressor T cells, were never fully characterised and gradually interest in this field declined. Today the area of regulatory T cells as they have become known, has seen a massive explosion of interest following a seminal study carried out by Sakaguchi *et al.* in 1995. This work demonstrated that autoimmune disease could be induced in BALB/c athymic nu/nu mice following the adoptive transfer of CD4+CD25- T cells. Co-transfer of CD4+CD25+ T cells prevented the development of autoimmune disease (Sakaguchi *et al.*, 1995). These studies were subsequently supported by data generated *in vitro* showing that mouse CD4+CD25+ regulatory T

cells could inhibit proliferation of CD4+CD25- T cells following stimulation with anti-CD3 monoclonal antibody (mAb) (Thornton and Shevach, 1998). Interestingly, the same group also showed that the co-culture of CD4+CD25- T cells isolated from the DO11.10 mouse strain in the presence of the relevant ovalbumin peptide, with CD4+CD25+ T cells isolated from the BALB/c mouse, failed to show suppression. However, suppression was restored following stimulation with anti-CD3, highlighting the requirement for activation of the CD4+CD25+ T cells through the TCR. Together these early studies in the mouse provide evidence that a specific population of CD25 expressing CD4+ T cells with regulatory function, appear to exist within the lymphocyte pool.

1.5.1 Origin of the CD4+CD25+ regulatory T cell

A fundamental question to be addressed is whether these CD4+CD25+ regulatory T cells represent a unique cell lineage, or whether all CD4+ T cells have the inherent ability to become regulatory T cells. There is evidence to suggest that these cells are indeed a dedicated cell lineage, arising naturally as a normal product of thymic selection. Neonatal thymectomy before day 3 after birth, results in the development of various autoimmune diseases in mice due to an absence of CD4+CD25+ cells (Suri-Payer *et al.*, 1998). Moreover, transfer of thymocyte suspensions depleted of CD4+CD25+ cells into syngeneic athymic nu/nu mice also results in the development of autoimmune disease (Itoh *et al.*, 1999). Historically a developing thymocyte was always thought to have one of three fates, either death by neglect, positive selection or negative selection, as already described. However, it now appears that there is a

potential fourth alternative fate which is dependent upon the thymocyte's interaction with MHC-self-peptide.

The current hypothesis suggests that CD4+CD25+ arise when there are relatively high-affinity interactions with self-peptide-MHC complexes, which are just below the threshold for negative selection. Experiments demonstrating this have been performed using mice expressing a transgenic TCR that recognises an influenza haemagglutinin (HA) peptide crossed to a lineage expressing the HA peptide (Jordan *et al.*, 2001). In these double transgenic mice, the HA-specific T cells were not deleted as one might have expected, instead approximately 50% of them were CD25+ and functioned as CD4+CD25+ regulatory cells. However, the use of transgenic mice expressing a TCR with lower affinity for the HA peptide, resulted in positive selection of CD4+ T cells but failed to generate any CD4+CD25+ regulatory cells. Conversely, when the high affinity HA-specific TCR-transgenic mouse was crossed with a transgenic line that expressed higher amounts of HA peptide, clonal deletion occurred. This narrow window of selection for the CD4+CD25+ regulatory cell ensures they represent only a small proportion of positively selected thymocytes, yet they are more sensitive to self-peptide-MHC than the potentially pathogenic autoreactive T cells.

Glucocorticoid (GC) signalling also supports the concept that TCR affinity is important for thymic selection of CD4+CD25+ T cells. GCs are immunosuppressants and by blocking their signalling, the threshold for T cell activation decreases, which mimics an increase in TCR signal strength. A novel study showed that by using GC receptor knockout mice and by blocking endogenous GC with drugs, the thymic T cell

repertoire was biased towards the production of CD4+CD25+ regulatory cells (Stephens and Ignatowicz, 2003).

At which stage in thymic development CD4+CD25+ cells are selected is not clear. The expression of CD25 suggests that this molecule may play a role in selection of these cells in the thymus. CD25 expression occurs relatively late in thymocyte development at the CD4+ single positive stage (Papiernik *et al.*, 1998). This suggests that if CD25 does play a role in the thymic selection of CD4+CD25+ regulatory T cells, the stimuli for the fourth alternative fate for T cell selection may occur in the medulla region of the thymus, either at the same time or after negative selection. Evidence that the interleukin (IL)-2 - IL-2 receptor (R) pathway may play a role in the selection of CD4+CD25+ regulatory T cells comes from the observation that IL-2, IL-2Rα (CD25) and IL-2Rβ (CD122) knockout mice all exhibit lethal lymphoproliferative disorders and autoimmunity (Sadlack *et al.*, 1993; Willerford *et al.*, 1995; Suzuki *et al.*, 1995). There have also been reports of a young male patient who has a genetic mutation in the CD25 gene and lacks the expression of cell surface CD25. This patient has a phenotype similar to mice deficient in IL-2 or the IL-2R, exhibiting severe lymphadenopathy and multiorgan inflammatory infiltrates (Roifman, 2000). This lack of T cell regulation led to speculation that this process was driven by the failure of IL-2 to sensitise autoreactive T cells to undergo activation-induced cell death (AICD). However, subsequent studies have now shown that the lack of regulation is due to defective regulatory T cells. Indeed, reconstitiution of CD4+CD25+ regulatory T cells into IL-2 knockout mice has been shown to prevent the development of lymphoproliferation and lethal autoimmunity (Malek and Bayer, 2004).

In related experiments, the adoptive transfer of wild-type CD4+CD25+ regulatory T cells into neonatal IL-2Rβ-/- mice prevented the development of lethal autoimmunity (Malek *et al.*, 2002). Also the thymic–specific expression of IL-2Rβ in IL-2Rβ-/- mice restored the production of functional CD4+CD25+ regulatory T cells (Malek *et al.*, 2000). The source of IL-2 required to drive this selection process may in part be T cell-independent; indeed DCs have been shown to produce IL-2 (Granucci *et al.*, 2001). It is likely that other pathways also play a role in the selection of CD4+CD25+ regulatory T cells. CD28-deficient and CD80/CD86 double-deficient mice also exhibit significantly diminished numbers of CD4+CD25+ regulatory cells, suggesting that CD28-mediated signals are also important in the development of CD4+CD25+ regulatory cells (Salomon *et al.*, 2000).

1.5.2 Human CD4+CD25+ regulatory T cells

Following the publication by Sakaguchi in 1995 identifying CD25 as a marker for mouse regulatory CD4+ T cells, it took a further six years before confirmation of an equivalent cell type in the human was published. The reasons for this delay might reflect a sceptical human immunological fraternity who were reluctant to be drawn back into the field of suppressor T cell biology. The role of T helper 1 (Th1) and Th2 biology was also very prominent at this time and researchers were attempting to identify these cells in human disease (Romagnani, 1995). Nevertheless a number of groups did investigate whether CD4+CD25+ T cells with regulatory activity were present in the human immune system. Studies using both human CD4+CD25+ thymocytes and peripheral blood derived CD4+CD25+ T cells confirmed that these

cells failed to proliferate and secrete IL-2 in response to TCR ligation, but were able to suppress the proliferative responses of both CD4+ and CD8+ T cells (Stephens *et al.*, 2001; Baecher-Allan *et al.*, 2001; Levings *et al.*, 2001; Ng *et al.*, 2001; Dieckmann *et al.*, 2001; Taams *et al.*, 2001; Jonuleit *et al.*, 2001).

In all of the studies the suppression was found to be dependent on cell-cell contact, confirming the observations seen with mouse CD4+CD25+ regulatory T cells *in vitro* (Thornton and Shevach, 1998). Further studies by Taams *et al.* showed that the CD4+CD25+ T cells expressed a highly diverse T cell repetoire and were able to recognise and respond to self antigens, dietary antigens and foreign antigens (Taams *et al.*, 2002). The use of thymocytes in these studies confirmed that the thymus also appeared to play a role in the generation of human CD4+CD25+ regulatory T cells. A more detailed investigation into the correlation between CD25 expression and regulatory activity showed that in humans, only the very highest expressing CD25+ T cells exhibit regulatory activity, often referred to as $CD25^{hi}$ (Baecher-Allan *et al.*, 2001).

1.5.3 Mode of suppression: cell-cell contact

How the CD4+CD25+ regulatory T cell mediates suppression is still an unresolved phenomemon. As already mentioned, *in vitro* studies have shown that cell-cell contact is paramount for suppression to occur, suggesting that a particular cell surface molecule may be responsible for this functionality. One candidate for this role is cytotoxic T lymphocyte-assoicated molecule-4 (CTLA-4). Both human and mouse derived CD4+CD25+ regulatory T cells express elevated levels of CTLA-4

suggesting a possible functional role for this molecule. Studies using a blocking antibody to mouse CTLA-4 have shown reversal of suppression mediated by CD4+CD25+ regulatory cells *in vitro* (Takahashi *et al.*, 2000). Treatment with an anti-CTLA-4 mAb also abrogated the ability of CD4+CD25+ T cells to inhibit colitis (Read *et al.*, 2000). Partial reversal of suppression has also been observed using a neutralising antibody to human CTLA-4 in cultures with human thymocyte-derived CD4+CD25+ T cells (Annunziato *et al.*, 2002).

However at present, the precise molecular mechanism underpinning CTLA-4 and the generation of regulatory T cell function remains unclear. Studies have shown that signalling through CTLA-4 may induce transforming growth factor beta (TGFβ) (Chen *et al.*, 1998). Alternatively, in a complex *in vivo* environment, the CD4+CD25+ regulatory cell may also interact directly with the APC *via* CTLA-4 on the CD4+CD25+ regulatory T cell and CD80 and CD86 on the surface of the APC. This interaction may induce the upregulation of indoleamine 2,3-dioxygenase (IDO) within the APC, resulting in tryptophan catabolism. This may then lead to immune suppression resulting from a reduction in CD4+CD25- T-cell function and viability due to tryptophan depletion and the production of pro-apoptotic metabolites. (Fallarino *et al.*, 2003; Grohmann *et al.*, 2003). It is also feasible that CTLA-4 may even act directly on the CD4+CD25- effector cell *via* interaction with CD80 or CD86 and initiate reverse signalling back into the effector cell, thus limiting T cell expansion (Paust *et al.*, 2004). Interestingly CD4+CD25- T cells isolated from CD80/CD86 doubly-deficient mice were resistant to suppression by CD4+CD25+ regulatory T cells *in vitro* (Paust *et al.*, 2004).

However, the role of CTLA-4 in CD4+CD25+ mediated suppression is still somewhat controversial as there are many reports of *in vitro* studies where antibodies to CTLA-4 have had no effect on suppression (Thornton and Shevach, 1998; Levings *et al.*, 2001; Baecher-Allan *et al.*, 2001). Also CD4+CD25+ cells from CTLA-4-/- mice have been shown to possess suppressive activity (Piccirillo and Thornton, 2004).

Another cell surface molecule that has recently generated great interest as a potential candidate for mediating cell-contact dependent suppression is glucocorticoid-induced tumour-necrosis factor receptor family-related protein (GITR). GITR was initially identified as a cell surface marker of mouse CD4+CD25+ regulatory T cells, following transcriptional gene profiling of CD4+CD25+ T cells versus CD4+CD25- T cells (McHugh *et al.*, 2002; Gavin *et al.*, 2002). An alternative method using immunisation with mouse CD4+CD25+ T cells and subsequent hybridoma screening, also identified GITR as a specific marker of CD4+CD25+ regulatory T cells (Shimizu *et al.*, 2002). Adoptive transfer studies have shown that the transfer of GITR-depleted CD4+ T cells into athymic mice resulted in a more aggressive autoimmunity than that observed following the adoptive transfer of CD25-depleted CD4+ T cells (Uraushihara *et al.*, 2003). However, studies showing a direct role for this molecule in mediating regulatory function are lacking. Indeed *in vitro* studies using an agonising antibody to GITR resulted in the abrogation of suppressive activity by the CD4+CD25+ T cells (Shimizu *et al.*, 2002). Conversely, addition of a Fab' fragment of the antibody had no effect on the ability of the CD4+CD25+ T cell to regulate, therefore implying that GITR does not play a direct role in the suppressive mechanism. The GITR-deficient mouse also does not reveal any significant alterations in its thymic or peripheral T cell subsets and does not have any overt signs

of autoimmunity (Ronchetti *et al.*, 2002). In agreement with this, human T cells do not express GITR ligands (Gurney *et al.*, 1999) and whether human CD4+CD25+ T cells express elevated levels of GITR is also unclear.

A new molecule that has recently emerged as a potential contender for mediating cell contact-dependent suppression is lymphocyte activation gene 3 (LAG-3) or CD223 as it is also known. This molecule is an MHC class II binding homologue of CD4. Expression of LAG-3 is upregulated on the surface of mouse CD4+CD25+ regulatory T cells following activation. Ectopic expression of LAG-3 *via* retroviral transduction was able to confer regulatory activity to mouse CD4+CD25- T cells (Huang *et al.*, 2004). However the molecular mechanism of action of LAG-3 in unknown and its importance in human regulatory T cell function has yet to be determined.

Whilst it is an attractive hypothesis that there is a unique CD4+CD25+-associated molecule that is responsible for mediating suppression, it is possible that this is not the case. Instead the cell contact-dependent mediated suppression observed *in vitro* may be the result of different, already identified molecules, acting semi-redundantly together to generate a suppressive phenotype.

1.5.4 Mode of suppression: soluble mediators

In vitro analysis has given an insight into how CD4+CD25+ T cells might mediate suppression through cell-cell contact. There is evidence *in vitro* that suppression by CD4+CD25+ T cells can occur in the absence for example, of APCs. Indeed, studies with human CD4+CD25+ regulatory T cells have shown that they can suppress

CD4+CD25- T cells following stimulation with plate-bound anti-CD3 alone (Levings *et al.*, 2001). Also, preactivated CD4+CD25+ were able to suppress the proliferative response of TCR-transgenic CD8+ T cells stimulated with soluble peptide-MHC class I tetramers (Piccirillo and Shevach, 2001). The use of transwells to investigate a role for soluble mediators has suggested a redundant role for cytokines in mediating suppression *in vitro* (Ng *et al.*, 2001; Taams *et al.*, 2001; Jonuleit *et al.*, 2001; Dieckmann *et al.*, 2001). However, these systems can never fully replicate the situation *in vivo*. The mode of action of the CD4+CD25+ regulatory T cell is thus probably much more complex than that observed *in vitro*. Moreover, the *in vivo* environment allows the regulatory cell to interact with many different cell types, a mechanism the regulatory cell may employ to amplify its suppressive effects.

The fact that the situation *in vivo* is likely to be much more complex and very different to that observed *in vitro*, may in part explain some of the discrepancies involving the role of cytokines in mediating suppression. Both IL-10 and TGFβ have been shown to play a role in regulatory T cell mediated suppression *in vivo*. In a severe combined immunodeficiency (SCID) colitis model it has been shown that treatment with an anti-IL-10 receptor antibody, abrogated the suppressive function of $CD45RB^{low}$ cells (Asseman *et al.*, 1999). $CD45RB^{low}$ cells are a population of regulatory T cells that are predominantly CD25 positive too. Similarly, $CD45RB^{low}$ cells isolated from $IL\text{-}10^{-/-}$ mice, failed to protect against colitis when co-transferred with $CD45RB^{hi}$ cells (Asseman *et al.*, 1999). $IL\text{-}10^{-/-}$ CD4+CD25+ T cells were also unable to inhibit *Helicobacter hepaticus* (*H. hepaticus*)-induced intestinal inflammation or anti-leishmania immunity (Kullberg *et al.*, 2002; Belkaid *et al.*, 2002). Interestingly the role of IL-10 here appears to be linked to control of the innate

immune system, so IL-10 may only play a role when the innate immune system is triggered. A recent publication has shown that CD4+CD25+ are responsible for inhibiting the sustained activation of the innate immune system following *H. hepaticus* infection of immune-deficient mice through the secretion of IL-10 (Maloy *et al.*, 2003). This role for IL-10 in controlling the innate response may in part explain why CD4+CD25+ cells from IL-$10^{-/-}$ mice were fully competent suppressors *in vitro* (Thornton and Shevach, 1998).

Whether TGFβ plays a role in the mechanism of suppression by CD4+CD25+ regulatory cells is another area of much debate. Again in the SCID-colitis model of inflammatory bowel disease (IBD), neutralising antibodies to TGFβ were able to block regulatory T cell function (Powrie *et al.*, 1996; Fuss *et al.*, 2002). However, in a model of gastritis, TGFβ was found to play no role in mediating regulatory T cell function (Piccirillo *et al.*, 2002). In addition, CD4+CD25+ cells isolated from TGF$\beta^{-/-}$ mice were functional suppressors *in vitro* (Piccirillo *et al.*, 2002). A recently published study has shown that CD4+CD25+ regulatory T cells isolated from TGF$\beta^{-/-}$ mice were also able to inhibit colitis (Fahlen *et al.*, 2005). However, the administration of an anti-TGFβ antibody was still able to abrogate suppression by these cells. Interestingly the transfer of CD4+CD45RB^{hi} cells expressing a dominant negative form of the TGFβ receptor type II, were resistant to suppression by CD4+CD25+ regulatory T cells. These studies show that whilst the production of TGFβ by CD4+CD25+ regulatory T cells is not essential for their function, it does play a role in their ability to suppress. Together these studies suggest that the CD4+CD25+ regulatory T cell may somehow induce TGFβ secretion from different cell types that may be haematopoietic or stromal in origin.

The concept that the CD4+CD25+ regulatory cell induces the expression of TGFβ by other cell types, is supported by studies *in vitro* demonstrating the phenomenon of infectious tolerance. CD4+CD25+ cells have the ability to induce conventional T cells to become regulatory T cells through the release of the immunomodulatory cytokines IL-10 and TGFβ (Dieckmann *et al.*, 2002;Jonuleit *et al.*, 2002). A recent study has further subdivided the CD4+CD25+ regulatory T cell into two different subsets defined by the expression of the integrins α4β7 or α4β1. They have shown that the α4β7 expressing regulatory cells induce IL-10 producing regulatory T cells and the α4β1 expressing regulatory cells induce TGFβ producing regulatory T cells (Stassen *et al.*, 2004). The authors speculate that that the expression of different integrins by CD4+CD25+ regulatory T cells ensures that they traffic to the correct tissues *in vivo* to control inflammation.

1.5.5 Extrathymic generation of CD4+ regulatory cells

The concept of infectious tolerance is an attractive way to explain how relatively few CD4+CD25+ T cells can be effective at suppressing an immune response *in vivo*, and it also introduces the concept of inducible regulatory T cells, which are not conventionally selected in the thymus.

The two major classes of inducible regulatory T cells are the T regulatory type 1 (Tr1) and the Th3 cell. The Tr1 cell is typically characterised by the production of IL-10 and the Th3 cell by the production of TGFβ. These cells can be generated using a variety of different methods, however one common factor in their generation is that they receive sub-optimal signals preventing optimal T cell activation. Tr1 cells were

initially generated by the chronic stimulation of naïve CD4+ T cells in the presence of IL-10 (Groux *et al.*, 1997). These Tr1 cells are functionally suppressive *in vivo* and have been shown to inhibit both colitis and antigen specific IgE responses (Groux *et al.*, 1997; Cottrez *et al.*, 2000). Tr1 cells can also be generated *in vivo* by repeated exposure to antigen in a non-proinflammatory environment such as intranasal delivery of peptide. This has been demonstrated with Tg4 transgenic mice specific for a myelin basic protein (MBP) peptide. Multiple intranasal doses of peptide were efficient at protecting the mice from developing experimental autoimmune encephalomyelitis (EAE) following subcutaneous injection of spinal cord homogenate in complete Freund's adjuvant (CFA) (Burkhart *et al.*, 1999). This effect was dependent on IL-10, as administration of an anti-IL-10 antibody to peptide-treated mice, resulted in the mice developing EAE. Further characterisation of the IL-10 producing cells confirmed they were antigen-specific Tr1 cells (Sundstedt *et al.*, 2003). Interestingly the ability of the Tr1 cells to suppress naïve Tg4 cells *in vitro* was not dependent on IL-10.

There are also reports of the generation of Tr1 cells in humans following peptide immunotherapy (Robinson *et al.*, 2004). Interestingly there is evidence that inducible regulatory cells may develop during a normal immune response to severe chronic infectious diseases. One such study has shown that pathogen-specific Tr1 cells were generated in the lungs of mice infected with *Bordetella pertussis* (*B. pertussis*). These cells were able to inhibit subsequent Th1 responses against *B. pertussis* both *in vitro* and *in vivo* (McGuirk *et al.*, 2002). Subsequent studies have shown that the lipopolysaccharide (LPS) from *B. pertussis* can bind to toll-like receptor (TLR) 4 on the surface of dendritic cells (DCs) and induce them to produce IL-10, which

facilitates Tr1 development (Higgins *et al.*, 2003). The generation of Tr1 cells in response to an invading pathogen was initially thought to be an evasion strategy by the pathogen to subvert protective Th1 responses. However now it is speculated that the generation of Tr1 cells are beneficial to the host as they limit immune pathology and potential tissue destruction within the lungs. Evidence to support this has been demonstrated by studies in mice which lack functional TLR4, where *B. pertussis* infection was more severe (Higgins *et al.*, 2003). There is also evidence to suggest that these cells occur as a natural population within the mouse gut where they regulate the responses to commensal flora (Cong *et al.*, 2002).

Th3 cells have been shown to be generated following oral administration of protein/peptide. In one study the oral administration of MBP, appeared to tolerise mice to MBP through the generation of Th3 cells, which suppressed the induction of EAE. This suppression was abrogated following the administration of an anti-TGFβ antibody (Chen *et al.*, 1994). Addition of TGFβ to murine T cell cultures has also been shown to promote the induction of Th3 cells (Weiner, 2001).

The APC may also play a role in the generation of inducible regulatory T cells. *In vitro* studies with human monocyte-derived DCs have shown that repetitive stimulation of allogeneic naïve cord blood cells with immature DCs, resulted in the generation of Tr1 cells (Jonuleit *et al.*, 2000). Another study has investigated the response in humans to the injection of antigen loaded immature DCs. A single injection of immature DCs pulsed with influenza matrix peptide (MP) resulted in the specific inhibition of MP-specific CD8+ T-cell effector function, i.e. cell killing and IFNγ production. This inhibition of T-cell effector function was accompanied by the

appearance of MP-specific IL-10 producing cells. Injection of mature DCs resulted in the restoration of CD8+ T-cell effector function (Dhodapkar *et al.*, 2001).

The lineage relationship of CD4+CD25+ regulatory T cells and Tr1 and Th3 cells is unclear. The CD4+CD25+ regulatory T cell is thought to derive from the thymus as a separate lineage. Conversely, the generation of Tr1 and Th3 cells is believed to occur in the periphery, derived from conventional CD4+CD25- naive T cells. The importance of each of the different regulatory T cell types in the regulation of immune responses is also unclear. What determines which cell acts when and where, is still to be resolved and is likely to be dependent on the microenvironment *in vivo* and the magnitude of the response. Whether the CD4+CD25+ regulatory T cell is the key orchestrater for the induction of these inducible regulatory cells also remains to be determined (Jonuleit *et al.*, 2002; Dieckmann *et al.*, 2002). The theory that CD4+CD25+ can transfer tolerising activity to induced regulatory cells is an attractive one and it is clear that *in vivo* the CD4+CD25+ regulatory T cell and the Tr1 and Th3 cells intimately co-operate to regulate immune responses effectively.

1.5.6 Clinical significance of CD4+CD25+ regulatory T cells in autoimmune disease

The central role of CD4+CD25+ regulatory T cells in controlling immune pathology in various mouse models of autoimmune disease and inhibiting T cell activation *in vitro*, is an accepted phenomenon. The central question is whether these cells have any clinical significance in human diseases. If these cells are responsible for controlling immune responses to self-antigens one might expect either defective or a

reduced frequency of CD4+CD25+ regulatory cells to be present in patients suffering from various autoimmune diseases.

Multiple sclerosis (MS) is an inflammatory demyelinating condition of the central nervous system where lymphocytes and macrophages dominate the inflammatory infiltrate. However, it is the T cell that is believed to be responsible for driving the autoaggressive response against the myelin components. Studies *in vitro* have shown that the magnitude of human CD4+ T cell proliferation in response to myelin oligodendrocyte glycoprotein (MOG) was increased following depletion of the CD4+CD25+ regulatory T cells. Conversely, the addition of CD4+CD25+ regulatory T cells to the cultures, inhibited MOG-specific proliferation (Wing *et al.*, 2003). These studies suggested that defective CD4+CD25+ T cells might play a fundamental role in the pathology of MS. Initial attempts to identify differences in CD4+CD25+ regulatory T cell frequencies between normal individuals and patients with MS, highlighted no significant variation (Putheti *et al.*, 2004).

A second study showed that although there was no change in frequency of CD4+CD25^{hi} T cells from patients and healthy controls, there was a marked decrease in the effector function of the CD4+CD25^{hi} T cells (Viglietta *et al.*, 2004). Following stimulation with sub-optimal levels of plate-bound anti-CD3 mAb, the level of T cell suppression was 80% with healthy control cells, but only 20% with MS patient-derived CD4+CD25^{hi} cells. In contrast, optimal stimulation with soluble anti-CD3 and anti-CD28 revealed no differences in the ability of the patient derived cells to suppress when compared to the normal controls at a 1:1 ratio. These experiments

demonstrate that the strength of signal delivered through both the TCR and costimulatory molecules, affects the levels of suppression achieved.

It is conceivable that this effect could be specific to MS, however other studies are now beginning to show defective regulatory T cells in other autoimmune diseases. Autoimmune polyglandular syndrome type II (AMPSII) is characterised by multiple endocrine diseases like type I diabetes and autoimmune thyroid disease. A recent study has shown that the suppressive capacity of the CD4+CD25+ regulatory T cells isolated from the blood of patients suffering from AMPS II was defective. However there were no obvious quantitative or phenotypic differences between the CD4+CD25+ regulatory T cells isolated from patients and controls (Kriegel *et al.*, 2004). Functional assays conducted using CD4+CD25^{hi} regulatory T cells isolated from the blood of patients with psoriasis also confirmed a defect in their suppressor capacity (Sugiyama *et al.*, 2005). Importantly, each of the human *ex vivo* studies mentioned thus far, confirmed through the use of co-mixing experiments, that the defective suppressor capacity resided within the CD25+ population and not the responder CD25- population. Indeed, defective regulatory T cells in psoriasis have been confirmed by a second study showing reduced suppressive capacity (Viglietta *et al.*, 2004).

An interesting study using CD4+CD25+ regulatory T cells isolated from the blood of patients with active rheumatoid arthritis (RA) has shown that the regulatory cells were able to suppress the responder CD4+ proliferative response, but failed to inhibit the secretion of the pro-inflammatory cytokines, TNFα and IFNγ (Ehrenstein *et al.*, 2004). Interestingly, isolation of CD4+CD25+ regulatory T cells from the same group

of patients after therapy with anti-TNFα mAb (Infliximab) showed that the regulatory T cells had regained the ability to inhibit TNFα and IFNγ production. However, the addition of TNFα to the coculture system was unable to overcome this suppressive effect and conversely, *in vitro* neutralisation of TNFα was not sufficient to restore the defect in the CD4+CD25+ population. The CD4+CD25+ regulatory T cells isolated from the RA patients prior to treatment with anti-TNFα mAb, were also unable to induce infectious tolerance in CD4+CD25- T cells *in vitro*, but this was restored following treatment with anti-TNFα mAb. The molecular mechanism by which Infliximab modulates CD4+CD25+ regulatory T cell function remains to be elucidated.

Although the numbers of regulatory T cells in each of the autoimmune diseases described thus far have revealed no significant differences from healthy controls, there are reports of reduced numbers of CD4+CD25+ regulatory T cells in systemic lupus erythematosus (SLE) but there is no functional data (Crispin *et al.*, 2003; Liu *et al.*, 2004). To date there are no reports of dysfunctional CD4+CD25+ regulatory T cells from patients with inflammatory bowel disease (IBD), indeed one study has shown strong suppressive activity of regulatory cells isolated from the blood of patients with IBD (Viglietta *et al.*, 2004). Clearly the role of regulatory T cells in controlling autoimmune pathologies is an area of great interest and further detailed studies involving monitoring the effects of various therapies on this regulatory T cell subset will help define their pathogenic role in human autoimmune disease.

1.5.7 Clinical significance of CD4+CD25+ regulatory T cells in cancer

The presence of functional CD4+CD25+ regulatory T cells is believed to contribute to the maintenance of peripheral tolerance by suppressing autoreactive T cells, thus preventing autoimmunity. Conversely, the failure of host anti-tumour immunity may be caused by the effective suppression of tumour-associated antigen-reactive lymphocytes, mediated by CD4+CD25+ regulatory T cells. A study using CD4+CD25+ regulatory T cells isolated from lung cancer patients showed that the regulatory cells were effective at inhibiting autologous peripheral blood mononuclear (PBMC) proliferation, following stimulation with either anti-CD3 alone or anti-CD3 in combination with anti-CD28. The study also showed there were large numbers of regulatory T cells infiltrating into the tumours and they expressed high levels of surface CTLA-4 (Woo *et al.*, 2002). Elevated numbers of CD4+CD25+ regulatory T cells have also been identified within the tumour-infiltrating lymphocyte population in patients with gastric and eosophageal cancers (Ichihara *et al.*, 2003).

A recent study of ovarian cancer has found large numbers of CD4+CD25+ regulatory T cells in the malignant tumours and ascites. The isolation of these cells from the tumour ascites confirmed the ability of the cells to inhibit the proliferation of autologous responder T cells stimulated *in vitro* with myeloid dendritic cells pulsed with Her2 peptides. This effect was replicated *in vivo* with mice receiving primary human ovarian tumour cells to establish tumour formation. Twelve days later the mice were given either autologous tumour specific T cells alone, or in combination with tumour ascites derived CD4+CD25+ T cells. The mice that received the tumour specific T cells in combination with the CD4+CD25+ T cells, showed progressive

tumour growth compared to the group that received the tumour specific T cells alone (Curiel *et al.*, 2004). These data indicate that tumour-derived CD4+CD25+ regulatory T cells may indeed hamper tumour-specific effector T cell immunity in individuals with cancer. Moreover, following analysis of 70 patients with ovarian cancer there was a significant negative relationship between number of CD4+CD25+ T cells present in the tumour tissue and survival.

1.5.8 Clinical significance of CD4+CD25+ regulatory T cells in allergic disease

Atopic allergic sensitisation is defined by the production of IgE against various innocuous environmental antigens such as grass pollen, house dust mite and animal dander. This can lead to the development of asthma, rhinitis and atopic dermatitis (Kay, 2001). The switch by B cells to produce IgE and the accumulation of eosinophils are under the control of IL-4 and IL-5, produced by Th2 cells. Studies *in vitro* have confirmed that human CD4+CD25+ regulatory T cells are effective inhibitors of Th2 mediated responses (Tiemessen *et al.*, 2002; Bellinghausen *et al.*, 2003). One possible explanation for the development of atopy is the failure of the CD4+CD25+ regulatory T cell to effectively regulate this population of cells. A recent study has shown that the suppressive ability of CD4+CD25+ regulatory T cells from atopic subjects in co-cultures with allergen-stimulated autologus CD4+CD25- T cells, was significantly reduced as compared to CD4+CD25+ regulatory T cells isolated from non-atopics (Ling *et al.*, 2004). Interestingly, the CD4+CD25+ regulatory T cells from the atopics were able to regulate following stimulation with anti-CD3 and anti-CD28.

Tr1 cells are also thought to play an important role in the prevention of atopic sensitisation. Indeed T cells engineered to secrete IL-10 were shown to greatly reduce the development of airway hyperactivity and airway inflammation (Oh *et al.*, 2002). One study has shown an increased number of allergen-specific IL-10-producing CD4+ T cells in the blood of non-atopics as compared to atopics and these Tr1 cells were effective at inhibiting IL-4 production by allergen-stimulated T cells *in vitro*. This suppression was reversed following the addition of a blocking antibody to IL-10 (Akdis *et al.*, 2004).

1.5.9 Clinical significance of CD4+CD25+ regulatory T cells in infectious diseases

As already mentioned, the TCR is believed to be highly cross-reactive. Experiments conducted *in vitro* using tetanus toxoid and grass pollen as the stimulating antigens, have confirmed the ability of human CD4+CD25+ regulatory T cells to suppress the response of CD4+CD25- effector T cells to both antigens (Taams *et al.*, 2002; Ling *et al.*, 2004). These studies highlight the ability of regulatory T cells to respond to foreign antigens. However, this observation does not necessarily mean that these cells participate in immune responses. Indeed it was previously thought that regulatory T cells only inhibited responses to self-antigens thus preventing autoimmunity, whilst permitting T cell responses against pathogenic organisms to proceed unchecked. This view is now being challenged by data that suggests that far from being uninvolved, CD4+CD25+ regulatory T cells do appear to participate in the immune response following pathogenic invasion.

The induction of pathogen-specific responses is crucial in host protection and resolution of potentially fatal disease processes. However these same effector mechanisms that have evolved to protect the host from the invading pathogen, can also cause immune-mediated pathology such as severe inflammation and tissue damage, if not tightly regulated. Evidence that CD4+CD25+ regulatory T cells play a role in infectious diseases has arisen mainly from mouse models of disease. One of the best characterised is infection with the parasitic pathogen, *Leishmania major* (*L. major*). Infection of C57BL/6 mice with *L. major*, results in a persistent low level of infection at the cutaneous infection site. Depletion of the CD4+CD25+ regulatory T cells resulted in clearance of the pathogen. However, as a consequence of the removal of the regulatory T cells, the mice did not establish lasting immunity and were susceptible to subsequent re-infection. In this model the presence of CD4+CD25+ regulatory T cells is beneficial to the host because although the parasite is allowed to survive, the persistence of the parasite confers immunity on the host to reinfection (Belkaid *et al.*, 2002). CD4+CD25+ regulatory T cells have also shown beneficial effects in mouse strains that are susceptible to *L. major* infection by limiting the extent of tissue damage (Aseffa *et al.*, 2002).

Conflicting data is emerging to suggest that CD4+CD25+ regulatory T cells may also play a role in the pathogenesis of human immunodeficiency virus (HIV) in humans. HIV infection is characterised by a loss of CD4+ T cells and progressive CD4+ T cell immune dysfunction, leading to impaired HIV-specific responses. One study has suggested that the presence of functional CD4+CD25+ regulatory T cells acts to the detriment's of the host following infection with HIV. Depletion of CD4+CD25+ regulatory T cells from peripheral blood samples taken from patients with HIV,

enhanced subsequent HIV T cell-specific responsiveness *in vitro* (Aandahl *et al.*, 2004). A second group have shown that infection of monocyte-derived DCs with HIV inhibited DC maturation, resulting in the production of IL-10 and induced regulatory T cells (Granelli-Piperno *et al.*, 2004).

Conflicting results were reported in a third study, which suggested that the decrease in response to HIV is due to defective CD4+CD25+ regulatory T cells. In the majority of healthy yet HIV-infected individuals, the CD4+CD25^{hi} T cells were able to suppress cellular proliferation and cytokine production by both CD4+ and CD8+ T cells, following stimulation with HIV antigens/peptides *in vitro*. These patients also exhibited lower levels of plasma viraemia as compared to patients without regulatory T cell activity. The authors suggested that suppression of CD4+ T cell activation by the regulatory cells is beneficial to the host because it limits the level of viral replication, thus reducing the number of target cells available for the virus to infect (Kinter *et al.*, 2004).

Clearly the role of CD4+CD25+ regulatory T cells in controlling infectious disease is complex and controversial, although these cells do appear to play an essential role by limiting the extent of immunopathology. However, there are also instances where the presence of CD4+CD25+ regulatory T cell may be detrimental to the host by suppressing anti-pathogen protective responses.

1.5.10 Therapeutic application of regulatory T cells

Both the 'naturally occurring' CD4+CD25+ regulatory T cell and the extrathymically generated Tr1 or Th3 regulatory T cells are thought to play important roles in controlling the immune response. There is increasing evidence that these cells play important roles in human pathologies, suggesting that the therapeutic manipulation of these cells could be beneficial in treating various human diseases.

To be of clinical benefit however, CD4+CD25+ regulatory T cells must be capable of inhibiting established disease. A study in the SCID/CD45RB^{hi} mouse transfer model of colitis has shown that following establishment of disease with CD45RB^{hi} cells, the adoptive transfer of CD4+CD25+ regulatory T cells was able to reverse disease (Mottet *et al.*, 2003). Whilst validating the concept that CD4+CD25+ regulatory T cells might be effective at treating autoimmune diseases, translating this into a successful therapy is somewhat more difficult. Boosting intrinsic CD4+CD25+ regulatory T cell activity or stimulating expansion of these cells *in vivo* may be one option for therapeutic intervention. Interestingly *in vitro* exposure of human CD4+CD25+ regulatory T cells to corticosteroids has been shown to increase their suppressive capacity following subsequent stimulation by allergen (Dao and Robinson, 2004). As already mentioned, treatment with anti-TNFα mAb, appeared to restore a defect in the suppressive capacity of the CD4+CD25+ regulatory T cell population isolated from patients with RA (Ehrenstein *et al.*, 2004).

Whilst treatment of autoimmune diseases may require expansion of the CD4+CD25+ regulatory T cell population, boosting anti-tumour immunity might be achieved

through the selective depletion of CD4+CD25+ regulatory T cells. Indeed several studies have shown that depletion of CD25+ positive cells *in vivo* using a depleting anti-CD25 antibody, induced the rejection of immunogenic tumours (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999). Currently trials of a product called Ontak are underway in advanced cancer patients. Ontak is a recombinant cytotoxic protein composed of portions of the diptheria toxin and the binding domain of human IL-2. Ontak binds to CD25+ T cells and after internalisation, causes cell death (Kreitman, 2003). Other potential targets include blocking chemokine receptors to stop trafficking of the CD4+CD25+ regulatory T cells into the tumour sites. A potential candidate is the chemokine receptor CCR4 or its ligand CCL22. *In vivo* studies in the NOD/SCID mouse reconstituted with primary ovarian human tumours have shown that human CD4+CD25+ regulatory cell migration but not CD4+CD25- T cell migration, into tumour tissues is dependent upon CCL22 and can be blocked with an antibody to CCL22 (Curiel *et al.*, 2004). Depletion of CD4+CD25+ regulatory T cells might also have beneficial effects in enhancing vaccination efficiency against persistent infections like HIV and hepatitis C.

Whilst the CD4+CD25+ regulatory T cell is an attractive target, some of the most promising therapeutic effects have arisen from studies looking at the generation of adaptive regulatory cells in the periphery. Initial studies focussed on treating allergic diseases with peptides to the sensitising allergen. Studies using either peptides of Fel d 1, a major cat allergen or phospholipase A_2 (PLA_2) peptides, from bee venom allergen, showed that following intradermal administration, patients were clinically hyporesponsive following challenge with the whole allergen (Oldfield *et al.*, 2001; Muller *et al.*, 1998). Following immunotherapy, PBMCs were isolated from the

treated patients and cultured *in vitro* in the presence of the peptides. Analysis of the cultures showed reduced levels of CD4+ T cell proliferation and both Th1 and Th2 cytokine production. However there was a significant increase in IL-10 production, suggesting the generation of Tr1 cells. A further study using Fel d 1 peptides showed that the allergen-specific T cells did undergo functional modifications, resulting in the generation of a population of regulatory T cells that were able to suppress antigen-induced proliferation of effector T cells (Verhoef *et al.*, 2005).

The success of peptide immunotherapy in treating allergic diseases has led to studies looking at treating autoimmune diseases using the same approach. Clinical trials involving oral administration of myelin basic protein (MBP) in MS, collagen in RA, thyroglobulin in autoimmune thyroid disease and S-antigen in uveitis have been conducted (von Herrath and Harrison, 2003). Disappointingly, none of the trials resulted in any significant clinical benefit. There are many possible explanations for the lack of efficacy observed including the route of administration, the dose of antigen, the choice of antigen and the timing of administration. Also patients with late stage disease may be less responsive to immunotherapy than patients at an early stage of disease, due to increased numbers of auto-reactive T cells. However, a new study looking at eptiope-specific immunotherapy in patients with early stage RA has shown some encouraging results. Following oral administration of dnaJP1, a bacterial heat shock protein, subsequent analysis of patient PBMCs revealed a significant increase in the number of IL-10 and IL-4 producing T cells, following *in vitro* peptide stimulation. There was also a significant decrease in the levels of proliferation and IL-2, IFNγ and TNFα production (Prakken *et al.*, 2004). Although this study was not

investigating clinical efficacy, patients on the trial showed a marked improvement from baseline and the therapy was found to be safe.

Clearly modulation of regulatory T cell function *in vivo* provides an exciting new opportunity in the treatment of various human diseases. However there are many facets of CD4+CD25+ regulatory T cell biology that are poorly understood including the antigenic specificity of the CD4+CD25+ regulatory T cell, the mechanism of suppression, the *in vivo* targets of the cells and the relationship between the naturally occurring CD4+CD25+ regulatory T cell and the adaptive regulatory T cell populations. The issues represent challenges for regulatory T cell specific immunotherapies. However an exciting new molecule has been discovered which may be a target for immunotherapy. Compounds that either inhibit this molecule or activate it may have enormous therapeutic potential in controlling CD4+CD25+ regulatory T cell function. This molecule, called FOXP3, identified as defective in the scurfy mouse (Brunkow *et al.*, 2001) has emerged as a leading contender for targeted therapies.

1.6 The Biology of FOXP3

1.6.1 The scurfy mouse

The original scurfy mutation occurred spontaneously in a partial inbred stock of mice at the Oak Ridge National Laboratory, USA in 1949. The discovery that the mutation was X-linked recessive provided early information on the sex-determining mechanism in mice (Russell, 1959). However it is the phenotype of the scurfy mouse that is of

interest to the immunologist. The scurfy mutation manifests itself as a severe lymphoproliferative disease characterised by runting, exfoliative dermatitis, thickening of the ears, squinted eyes, lymphadenopathy, splenomegaly and severe anaemia. These symptoms are so severe that death usually occurs between 15-24 days of age (Godfrey *et al.*, 1991). Studies using either anti-CD4 or anti-CD8 depleting antibodies to treat neonatal scurfy mice, have demonstrated that the effector cells responsible for development of the disease are CD4+ T cells (Blair *et al.*, 1994). Indeed the adoptive transfer of CD4+ but not CD8+ T cells from scurfy mice into nude mice transmitted the disease (Blair *et al.*, 1994).

Analysis of cytokine gene expression in the spleen, lymph nodes and skin of scurfy mice revealed dysregulated overexpression of various cytokine genes including IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFNγ and TNFα (Kanangat *et al.*, 1996). Further cellular characterisation of scurfy mice compared to normal littermates revealed differences in the numbers of macrophages and B cells. The onset of the disease was accompanied by a progressive increase in the numbers of Mac1+ cells and a concomitant decrease in the numbers of B220+ cells found within the spleen and lymph nodes (Clark *et al.*, 1999). There was also an increase in the number of CD4+ T cells and these appeared to be larger than CD4+ T cells from normal mice. The expression levels of the cell surface markers CD69, CD25, CD80 and CD86 were also elevated on the CD4+ T cells. Following stimulation through the TCR, the CD4+ T cells produced levels of GM-CSF more than 1000-fold greater than normal controls.

One interesting observation was the apparent hyper-responsiveness of the CD4+ T cells to TCR signalling. Lymph node cells freshly isolated from scurfy mice

underwent significant levels of proliferation even in the absence of TCR stimulation. Purification of the CD4+ T cell population and stimulation with increasing concentrations of plate-bound anti-TCRβ, showed that the CD4+ T cells were highly responsive to low levels of antibody as compared to normal controls. These cells also had a decreased requirement for costimulation through CD28. Interestingly these cells were highly refractory to inhibition by Cyclosporin A and showed reduced sensitivity to genistein and herbimycin A. This suggests some kind of impaired TCR signalling cascade which lowers the threshold for activation and then fails to downregulate the T cell activation response.

Although the *in vitro* T cell experiments described have shown a reduced threshold for T cell activation, an elegant study in the mouse showed the absolute requirement for stimulation through the TCR for the scurfy phenotype to manifest itself. Scurfy mice crossed with the DO11.10 strain and bred on a recombinase activator gene (RAG) 1 knockout background so that 100% of the T cells are reactive to OVA peptide, did not develop disease (Zahorsky-Reeves and Wilkinson, 2001) suggesting that in the scurfy mouse, it is the recognition of endogenous self-antigens or environmental antigens, which drives pathology.

1.6.2 Identification of the gene responsible for the scurfy phenotype

The gene responsible for the scurfy phenotype was identified using a classic positional gene cloning approach (Brunkow *et al.*, 2001). Sequence analysis identified the gene as a novel member of the forkhead/winged-helix family of transcriptional regulators and was designated *Foxp3*. The forkhead/winged helix family of

transcription factors comprises a large and diverse group of transcription factors that share a 'winged helix' DNA binding domain of approximately 110 amino acids. Over 100 proteins with forkhead domains have been identified, comprising at least 17 different subclasses (Jonsson and Peng, 2005). These proteins are believed to act as both transcriptional activators and repressors (Coffer and Burgering, 2004). The nomenclature is such that genes that encode human transcription factors are denoted by uppercase letters, i.e. *FOX*, whereas only the first letter is capitalised for genes that encode mouse transcription factors (*Fox*). Both human and mouse proteins are denoted by uppercase letters, i.e. FOX (Coffer and Burgering, 2004).

Unlike most members of the FKH family, FOXP3 is unique in having the functional FKH domain located near the C-terminus. Other interesting structural motifs in the FOXP3 protein include a proline-rich region at the amino terminus, a zinc finger and a leucine zipper (Gambineri *et al.*, 2003). The significance and function of these domains is currently unknown. The *Foxp3* gene in the scurfy mouse has a 2 base-pair insertion just upstream of the FKH domain, resulting in a frameshift mutation and a premature stop codon. This results in a truncated gene product lacking the functional C-terminal FKH domain, which suggests an important role for this domain in controlling T cell activation.

1.6.3 Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)

Although the human FOXP3 protein at 431 amino acids in size is 2 amino acids larger than the mouse FOXP3 protein, there is a high degree of sequence identity between

the two proteins with an overall similarity index of 86%. However within the FKH domain this rises to 94%, highlighting the importance of this domain to the functionality of the protein (Brunkow *et al.*, 2001). As the scurfy mutation is so severe, one might expect mutations in this region to also manifest as severe disease in humans. Indeed this is the case with evidence that mutations within the FOXP3 gene in humans cause IPEX (Bennett *et al.*, 2001; Wildin *et al.*, 2001).

IPEX is an X-linked fatal, recessive disorder that presents most commonly in early childhood. Enteropathy is the most common feature and patients often develop Crohn's disease or ulcerative colitis. Endocrinopathies including diabetes and thyroid abnormalities are also common, as are skin disorders and increased susceptibility to infections (Gambineri *et al.*, 2003). Patients also have high serum IgE levels and eosinophilia is sometimes present. There are also elevated levels of autoantibodies against different organs. Current therapy for IPEX patients involves the use of broad immunosuppressives like cyclosporin A and FK506, either alone or in combination with steroids. However these drugs are very toxic and long-term remission is never achieved. Bone-marrow transplantation is currently the only effective cure.

The mutations that cause IPEX within the human *FOXP3* gene are spread throughout the gene. Currently 13 different mutations have been reported within the *FOXP3* gene. The majority of the mutations are missense mutations within the FKH domain (Bennett *et al.*, 2001; Wildin *et al.*, 2001; Wildin *et al.*, 2002; Kobayashi *et al.*, 2001). There are also mutations affecting the leucine-zipper (Chatila *et al.*, 2000), the proline-rich domain (Kobayashi *et al.*, 2001) and there have been reports of deletions resulting in the removal of the stop codon, leading to proteins with C-terminal

extensions (Bennett *et al.*, 2001; Wildin *et al.*, 2001). Interestingly another report describes a patient who does not have a mutation within the coding region of the gene and exhibits a less severe form of the disease (Wildin *et al.*, 2001). The authors suggest that this patient may have a mutation in the non-coding region, which affects transcriptional regulation or RNA splicing. However, a recent report shows that in a cohort of 27 affected males from 24 families who were referred for sequencing of FOXP3 because of their clinical phenotype, only 60% had identifiable mutations within the coding region (Ochs *et al.*, 2005). A further 10% exhibited low mRNA levels suggesting possible mutations in the non-coding region. The remainder could have mutations within the promoter region or alternatively, they may have mutations in other genes which directly or indirectly interact with the functionality of FOXP3.

1.6.4 Function of FOXP3

The severity of the phenotype of both the scurfy mouse and patients with IPEX highlights the essential role of this protein in maintenance of immune homeostasis. The first insight into how FOXP3 acts to regulate CD4+ T cell function came from a study in Jurkat T cells (Schubert *et al.*, 2001). Overexpression of the protein in Jurkat T cells resulted in a dramatic reduction in the levels of IL-2 production. Confirmation that the FKH region of the protein is essential for function was demonstrated using an IL-2 reporter gene assay. A multimer of the essential regulatory nuclear factor activated T cells (NFAT) site from the mouse IL-2 promoter was cloned upstream of the luciferase reporter gene. Cotransfection of this construct with either FOXP3 or a mutant form of the gene lacking the FKH domain into Jurkat T cells and subsequent activation with phorbol-12-myristate, 13-acetate (PMA) and ionomycin, resulted in a

significant reduction in the levels of luciferase production by the cells expressing full-length FOXP3. This was also confirmed using the proximal IL-2 promoter to drive luciferase expression. These studies suggest that FOXP3 is a transcriptional repressor and may bind directly to regions within the promoter/enhancer elements of certain genes. Indeed further studies by the same group identified potential FKH binding sites adjacent to critical NFAT elements in several cytokine promoters.

The phenotype of the scurfy mouse is one of dysregulated T cell function and elevated cytokine production, supporting a role for FOXP3 as a transcriptional repressor of certain cytokine genes. To study the function of FOXP3 in more detail, several different *Foxp3*-transgenic mouse lines were established (Khattri *et al.*, 2001). Whilst expression of full-length FOXP3 protein prevented disease in each scurfy mutant mouse line, analysis of the T cell component of these mice revealed differences in T cell number dependent upon the number of copies of the transgene. Each of the lines showed reductions in the numbers of splenic and lymph node cells compared to healthy litter mate controls, with both CD4+ and CD8+ T cell numbers reduced. However this was most dramatic in the mouse lines expressing the highest copy number. Interestingly, transgenic animals that expressed the *Foxp3* gene exclusively in the thymus, were susceptible to disease. This observation highlights not only the requirement for expression of FOXP3 in the periphery, but also the fact that the scurfy syndrome is unlikely to arise as a consequence of developmental defects in the thymus.

Functional analysis of the CD4+ T cells isolated from *Foxp3* transgenic mice, revealed a significant reduction in the levels of proliferation and IL-2 production

compared to control animals, following activation *in vitro*. Interestingly the proliferative response of thymic CD4+ T cells, was comparable to those isolated from control mice. However the levels of IL-2 production were reduced. Analysis of the CD8+ T cells revealed that although the cells still possessed cytolytic activity, they were less effective than CD8+ T cells isolated from control mice. Together these data indicate that FOXP3 acts as a central regulator of T cell activity.

1.6.5 FOXP3 and CD4+CD25+ regulatory T cells

The studies described thus far have highlighted the importance of FOXP3 in controlling T cell activation. An insight into a role for FOXP3 in regulatory T cell function has come from the observation that the scurfy phenotype can be rescued following the adoptive transfer of wild-type T cell enriched splenocytes (Smyk-Pearson *et al.*, 2003). Female carriers of the *FOXP3* mutation are also apparently healthy. Even though they display random X chromosome inactivation, the presence of normal, in addition to mutated FOXP3 alleles, expressed in the peripheral CD4+ T cells of IPEX carriers, appears sufficient to prevent disease (Tommasini *et al.*, 2002). Together these observations are consistent with the theory that FOXP3 may play a role in regulatory T cell activity.

Evidence to support this theory has come from further studies with the *Foxp3* transgenic mouse (Khattri *et al.*, 2003). These authors showed that FOXP3 expression correlated with CD4+CD25+ T cells and was not upregulated in CD4+CD25- T cells following activation, suggesting that FOXP3 expression is specific to T cells of the regulatory T cell lineage. In addition, CD4+CD25+ T cells from scurfy mice lacked

regulatory activity whereas the CD4+CD25+ T cells from the *Foxp3* transgenic mice had suppressive activity. They further showed that the CD4+CD25- and CD8+ T cell populations but not the B cell population from the transgenic mouse also had suppressive activity.

Complementary to the studies by Khattri *et al.* (2003), two separate groups confirmed the specific expression of *Foxp3* in mouse CD4+CD25+ regulatory T cells. These studies showed that retroviral transduction of mouse CD4+CD25- T cells with the *Foxp3* gene results in the generation of regulatory T cell activity (Hori *et al.*, 2003; Fontenot *et al.*, 2003). Perhaps most impressively, these *Foxp3*-transduced cells were able to protect against colitis following transfer into a lymphopenic host together with pathogenic CD4+CD25- T cells. Following the publication of these studies, a subsequent study has shown that *Foxp3*-transduced CD4+ T cells with specificity for islet antigen were able to stabilise and reverse disease in mice with recent-onset diabetes (Jaeckel *et al.*, 2005).

Human CD4+CD25+ T cells have also been shown to express FOXP3 although interestingly in human cells, the protein appears to migrate as a doublet following immunoblot analysis (Walker *et al.*, 2003a). Overexpression of FOXP3 in human CD4+CD25- T cells following retroviral transduction was also sufficient to convert the cells into a regulatory phenotype (Yagi *et al.*, 2004). The cells were hyporesponsive following stimulation with soluble anti-CD3 mAb and autologous APCs, and were able to suppress the proliferative response of non-transduced CD4+CD25- T cells. Together these data highlight a significant role for FOXP3 in CD4+CD25+ regulatory T cell function.

1.6.6 FOXP3 and other T cell subsets

Whilst it would be tempting to conclude that FOXP3 is a truly specific marker for CD4+CD25+ regulatory T cells derived from the thymus, data is emerging to suggest this is not always the case. Activation of human CD4+CD25- T cells in the presence of plate-bound anti-CD3 mAb and soluble anti-CD28 mAb, resulted in the upregulation of FOXP3 expression in the CD25+ fraction of cells and conferred suppressive function upon the cells, which was cell-contact dependent (Walker *et al.*, 2003a). Further studies by the same group have shown that antigen-specific CD4+CD25+ regulatory T cells can be derived from CD4+CD25- T cells isolated from peripheral blood (Walker *et al.*, 2005). These data suggest that FOXP3 positive regulatory T cells may be generated in the periphery from CD4+CD25- T cells during an immune response, to help regulate the magnitude of the response. FOXP3 expression can also be upregulated in both human and mouse CD4+CD25- T cells following culture *in vitro* with TGFβ, resulting in the generation of suppressive activity (Fantini *et al.*, 2004). Expression analysis in human lymphocytes has also revealed that FOXP3 is not unique to CD4+ T cells. Alloantigen specific CD8+CD28- suppressor T cells also express FOXP3 (Scotto *et al.*, 2004). A recent paper has also shown that FOXP3 expression can be upregulated in CD8+ T cells following activation (Morgan *et al.*, 2005).

The relationship between naturally occurring CD4+CD25+ regulatory T cells and the extrathymically-derived Tr1 and Th3 regulatory T cells is still not clear. However, the expression profile of FOXP3 had suggested that the adaptive regulatory T cells lacked FOXP3 expression and thus were a separate lineage of regulatory T cells. Indeed

analysis of *in vitro* derived Tr1 regulatory T cells produced following stimulation with vitamin D_3 and dexamethasone, showed that although the cells did not express FOXP3, they were able to suppress the proliferation of CD4+CD25- responder T cells in the presence of anti-CD3 mAb and APCs. This suppression was cell-contact dependent and could not be reversed *via* the addition of blocking antibodies to IL-10, TGFβ or CTLA-4 (Vieira *et al.*, 2004). The *in vivo* generation of Tr1 cells following intranasal administration of a specific MBP peptide also generated cells that were FOXP3 negative (Vieira *et al.*, 2004). However a recent study has challenged this theory and shown that a population of antigen-specific regulatory T cells expressing FOXP3 and producing both IFNγ and IL-10, were generated following the adoptive transfer of antigen-pulsed mature CD8α dendritic cells (Stock *et al.*, 2004).

Whilst there is clearly a functional association with FOXP3 expression and regulatory T cell activity there are still many questions to be answered regarding the mechanism of action of FOXP3 and also the role of FOXP3 in generating both naturally occurring and adaptive regulatory T cells. Whether FOXP3 has functions outside of regulatory T cells also needs to be determined. Clearly this is an exciting area of research and as the biology of FOXP3 is unravelled it will hopefully provide new options for the treatment of human autoimmune diseases and cancer. Indeed there is already evidence to suggest that one of the beneficial mechanisms of action of glucocorticoids used for the treatment of asthma is to induce FOXP3 expression and increase regulatory T cell activity through the generation of Tr1 like cells. (Karagiannidis *et al.*, 2004).

1.7 Chimeric Receptors

The use of chimeric receptors to specifically target T cells to kill tumour cells through the antigen specific activation of T cells, is being considered as an immunotherapy approach to cancer (Sadelain *et al.*, 2003). This approach involves engineering a T cell to express a chimeric receptor consisting of an antibody-binding region linked *via* a transmembrane region to an intracellular signalling domain or domains, involved in T cell activation. The antibody-binding region generates the specificity and can be engineered to recognise different tumour antigens. Once the T cell encounters the antigen expressed on the surface of the tumour cell, the T cell will become activated resulting in tumour killing (Sadelain *et al.*, 2003).

Potent costimulatory signals can be delivered through the use of chimeric receptors with the intracellular signalling domain of CD28 linked in series to the intracellular signalling domain of the ζ-chain from the TCR complex (Finney *et al.*, 1998 and Figure 1.1). Stimulation of chimeric receptor transfected cells with solid phase antigen results in the production of IL-2, TNFα, IFNγ and GM-CSF (Finney *et al.*, 2004). Chimeric receptors providing costimulatory signals through other molecules such as CD134, ICOS and CD137 have also been described (Finney *et al.*, 2004). Whilst these molecules have been designed for use in targeted cancer immunotherapy, they also provide an effective means of specifically activating human T cells.

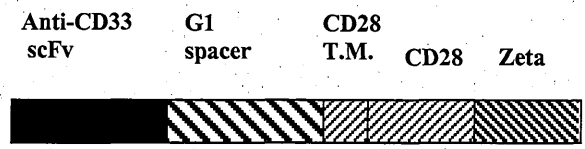


Figure 1.1 Schematic diagram of the CD28/TCRζ chimeric receptor.
The chimeric receptor consists of a single chain Fv specific for CD33, linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3. The spacer is linked to the transmembrane (T.M.) and intracellular regions of human CD28, which is linked to the intracellular region of human TCRζ.

The extracellular region of the receptor illustrated in figure 1.1, is an antibody single chain Fv (scFv) that binds to CD33. It has been reported that the addition of chimeric receptor transfected human T cells to CD33-coated antigen plates, induces a strong activating signal into the transfected cells through the scFv (Finney *et al.*, 2004). This mechanism of activation ensures that only the transfected cells receive stimulation. Production of cytokines can then be measured to give an indication of the levels of T cell activation.

1.8 Project Aims

Regulatory T cell biology has become a vast subject encompassing both naturally occurring CD4+CD25+ regulatory T cells and various inducible regulatory T cell types. Functional characterisation of these different cell types in terms of both cell surface marker expression and mode of action has produced differing results and conclusions. These discrepancies may partly be explained by species differences and also by the cellular environment within which the assays were conducted.

The primary aim of the studies described in this thesis was to carry out a thorough characterisation of human CD4+CD25+ regulatory T cells, derived from peripheral blood. A detailed analysis of the cell surface phenotype of freshly isolated and three day activated CD4+CD25+ regulatory T cells versus CD4+CD25- T cells, was carried out. Functional suppression assays were also established and the mechanism of suppression was investigated through the use of antibodies to various cell surface markers and cytokines, which have been implicated in regulatory T cell function.

The second aim of these studies was to investigate the function of FOXP3 following overexpression of the protein in both human CD4+ and CD8+ T cells. This was achieved through the establishment of a novel cotransfection system. This system utilised chimeric receptors to ensure that only transfected cells could receive activation signals. The final aim of the work presented in this thesis was to investigate the functionality of splice variant forms of the human *FOXP3* gene generated during the cloning of the gene. This was also carried out using the chimeric receptor system.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 General reagents

General laboratory chemicals were purchased from Sigma Aldrich Ltd, (Gillingham, U.K.) or BDH Ltd, (Poole, U.K.) unless stated otherwise. Ethanol and Methanol were from Hayman Ltd, (Witham, U.K.). Bacto-tryptone, bacto-agar and bacto-yeast extract were from Difco Ltd, (Detroit, U.S.A.).

All tissue culture media, supplements and biological buffers were supplied by Invitrogen Ltd, (Paisley, U.K.). Foetal calf serum (FCS) was from Helena Biosciences Ltd, (Sunderland, U.K.). Human AB serum was from Cambrex Biosciences Ltd, (Verviers, Belgium.). All tissue culture plastic ware was purchased from Falcon, BD Biosciences, (Cowley, U.K.) with the exception of 96-well U-bottomed plates which were purchased from Costar, (New York, U.S.A.) and ELISA plates which were Nunc^{TM} brand products supplied by Fisher Scientific, (Loughborough, U.K.). All cytokines were purchased from R&D Systems, (Abingdon, U.K.) with the exception of human IL-2 which came from Roche, (Lewes, U.K.). Tetanus Toxoid (TT) was purchased from CN Biosciences Ltd, (Nottingham, U.K.). CHO-K1 cells were from ECACC, (CAMR, Porton Down, U.K.).

The radionucleotide methyl-[^{3}H]- thymidine had a specific activity of 50μCi/mL and was purchased from Amersham Biosciences, (Chalfont St.Giles, U.K.).

Restriction endonucleases, dNTPs and DNA molecular weight markers were purchased from New England Biolabs Ltd, (Hitchin, U.K.). T4 DNA ligase (HC) and 10X ligation buffer were purchased from Promega, (Southampton, U.K.). DNA

sequences were cloned into pcDNA3.1+ or pTracer-CMV2 expression vectors, purchased from Invitrogen. QIAGEN Miniprep, Maxiprep, QIAquick PCR purification and gel extraction kits were all purchased from Qiagen Ltd, (Crawley, U.K.). Apparatus for horizontal gel electrophoresis was supplied by Invitrogen.

Big Dye Terminator Cycle Sequencing Ready Reaction DNA sequence kit was obtained from Applied Biosystems, (Warrington, U.K.). Sequencing reactions were carried out using a Biometra Trio-Thermablock (Anachem Ltd, Luton, U.K.). All other PCR reactions were carried out using a Primus 96 Plus PCR machine (MWG-Biotech, Milton Keynes, U.K.). Hot Start DNA polymerase was purchased from Novagen, (Nottingham, U.K.).

Tris-glycine SDS page gels and SeeBlue prestained molecular protein markers were supplied by Invitrogen. Immobilon-P PVDF membrane was from Millipore Ltd, (Watford, U.K.).

Small-scale centrifugation was performed using Eppendorf 5415D or Eppendorf 5415R refrigerated benchtop microcentrifuges (Anachem Ltd, Luton, U.K.). Sorvall RC3B and RC5B refrigerated centrifuges were used for large scale bacterial centrifugation. IEC Centra GP8R centrifuges were used for the centrifugation of mammalian cells.

2.1.2 Buffers and solutions

PBS	137mM NaCl 2.68mM KCl 10.1mM Na_2HPO_4 1.76mM KH_2PO_4 pH7.4
TAE	40mM Tris-acetate pH 8.0 2.5mM EDTA
TE	10mM Tris-HCl pH7.4 1mM EDTA
Hi TNE	65mM Tris-HCl 15mM EDTA 4M NaCl
5X DNA Loading buffer	0.25% (w/v) bromophenol blue 30% (w/v) glycerol
Membrane preparation buffer	20mM Hepes 10mM $MgCl_2$ 100mM NaCl, pH 7.4
6X protein sample buffer	60% glycerol 300mM Tris (pH 6.8) 12mM EDTA 12% SDS 864mM 2-mercaptoethanol 0.05% bromophenol blue
Electrophoresis running Buffer	24mM Tris base 192mM glycine 3.5mM SDS pH8.3

Tris-glycine transfer buffer	25mM Tris base 192mM glycine 20%(v/v) methanol
MACS Buffer	Sterile tissue culture grade PBS supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA. Sterile filtered and degassed.
Red cell lysis buffer	Sterile tissue culture grade distilled water supplemented with 155mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA. Sterile filtered.
LB Agar	1% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 171mM NaCl 1.5% (w/v) Bacto-agar pH7.0
SOC medium	2% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 8.6mM NaCl 25mM KCl 10mM $MgCl_2$ 20mM glucose pH7.0
2X TY broth	2% (w/v) Bacto-tryptone 1% (w/v) Bacto-yeast extract 171mM NaCl 1.5% (w/v) Bacto-agar pH7.0

2.1.3 Antibodies

The antibodies and fusion proteins used for functional assays are listed in table 2.1. All antibodies were used at 10μg/mL in functional assays, apart from the anti-CD3 antibodies which were used at 1μg/mL. The antibodies used for flow cytometric analyses are listed in table 2.2.

Specificity	Clone	Isotype	Function	Source
Hu IL-10	23738.111	mu IgG2b	neutralising	R&D
Hu TGFβ1,β2,β3	1D11	mu IgG1	neutralising	R&D
Hu IL-2	5334	mu IgG1	neutralising	R&D
Hu CD25	22722	mu IgG1	blocks ligand binding	R&D
Hu CD122	27302.1	mu IgG1	blocks ligand binding	R&D
Hu CTLA-4	ANC152.2/ 8H5	mu IgG1	blocks ligand binding	Ancell
Hu CD28	CD28.2	mu IgG1	costimulatory	BD
Hu CD134	L106	mu IgG1	blocks ligand binding	BD
Hu GITR	110416	mu IgG1	blocks ligand binding	R&D
Hu Lag-3	17B4	mu IgG1	blocks ligand binding	Apotech
Hu TGFβRII	polyclonal	goat IgG	blocks ligand binding	R&D
Hu IL-17	41809	mu IgG2b	neutralising	R&D
Hu TNFR2	22221	mu IgG2a	blocks ligand binding	R&D
Hu CD95	SM1/23	mu IgG2b	blocks apoptosis	Alexis
Hu CD95	APO-1-1	mu IgG1	Induces apoptosis upon cross-linking	Alexis
Hu CD3	OKT3	mu IgG2a	activation	Celltech
Mu CD3	145-2C11	hamster IgG1	activation	BD
CD22-muFc		mu IgG1	control fusion protein	Celltech
muCTLA-4muFc		mu IgG1	blocks ligand binding	Celltech
control	MOPC21	mu IgG1	control	Celltech
rat CD134	MAROX2A	mu IgG2a	control	Celltech
control	20116.11	mu IgG2b	control	R&D
control	polyclonal	goat IgG	control	R&D

Table 2.1 Antibodies and fusion proteins used in functional assays. Human is abbreviated to hu and mouse is abbreviated to mu.

Specificity	Clone	Isotype	Fluorochrome	Supplier	Conc used
Hu CD4	RPA-T4	Mu IgG1	FITC/PE	BD	5μL/10^5 cells
Hu CD8	HIT8a	Mu IgG1	FITC/PE	BD	5μL/10^5 cells
Hu CD25	M-A251	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD69	FN50	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD134	ACT35	Mu IgG1	PE	BD	5μL/10^5 cells
Hu ICOS	DX29	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD45RA	HI100	Mu IgG2b	PE	BD	5μL/10^5 cells
Hu CD45RO	UCHL1	Mu IgG2a	PE	BD	5μL/10^5 cells
Hu GITR	110416	Mu IgG1	PE	R&D	10μL/10^5 cells
Hu CD95	LOB3/17	Mu IgG1	PE	Serotec	5μL/10^5 cells
Hu TNFR2	22235	Mu IgG2a	PE	R&D	10μL/10^5 cells
Hu CTLA-4	BNI3	Mu IgG2a	PE	BD	5μL/10^5 cells
Hu CD103	LF61	Mu IgG1	PE	Serotec	5μL/10^5 cells
Hu CD27	M-T271	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD62L	DREG-56	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD122	Mik-β3	Mu IgG1	PE	BD	10μg/mL
Hu CD132	TUGh4	Rat IgG2b	PE	BD	10μg/mL
Hu CD40L	TRAP1	Mu IgG1	PE	BD	5μL/10^5 cells
Hu CD137	4B4-1	Mu IgG1	PE	BD	5μL/10^5 cells
Hu BDCA-4	AD5-17F6	Mu IgG1	PE	Miltenyi-Biotec	5μL/10^5 cells
Hu PD-1	MIH4	Mu IgG1	FITC	BD	10μg/mL
Hu Galectin-1	polyclonal	goat	unlabelled	R&D	10μg/mL
Mu CD4	YTS 177.9	Rat IgG2a	FITC	Serotec	5μL/10^5 cells
Mu CD25	PC61	Rat IgG1	PE	BD	10μg/mL
CD33 scFv		Hu protein	FITC/Alexa 488	Celltech	1μg/mL
Goat F(ab')$_2$	polyclonal	rabbit	FITC	Jacksons	2μg/mL
control	MOPC21	MuIgG1	PE/FITC	BD	5μL/10^5 cells
control	G155-178	MuIgG2a	PE	BD	5μL/10^5 cells
control	27-35	Mu IgG2b	PE	BD	5μL/10^5 cells
control	R35-38	Rat IgG2b	PE	BD	5μL/10^5 cells
control		Goat IgG	unlabelled	R&D	10μg/mL

Table 2.2 Antibodies and proteins used for flow cytometric analyses. Human is abbreviated to hu and mouse is abbreviated to mu.

2.1.4 Oligonucleotides

Oligonucleotides were designed using sequences in the Genbank nucleotide sequence database. Shown below are the cloning primers for human *FOXP3*, the flag-tagged FKH mutant and mouse *Foxp3*.

Human *FOXP3* – Genbank accession number AF277993:

huFOXP3FWD2 (forward)

5' – GAGAGA**GAATTC**GCCACC**ATG**CCCAACCCCAGGCCTGGCAA –3'

*Eco*RI Start

huFOXP3REV (reverse)

Stop

5' – GAGAGA**GCGGCCGCTCA**GGGGCCAGGTGTAGGGTTG – 3'

*Not*I

Human *FOXP3* flag-tagged FKH mutant:

huFOXP3FWD2 was used as the cloning forward primer.

FKH_FOXP3_REV (reverse)

Stop

*Hin*d III Flag sequence

5'- GAGAGA**AAGCTTCTA**CTTGTCATCGTCGTCCTTGTAGTCGTTGTGGAG GAACTCTGGGAATGTG – 3'

Mouse *Foxp3* – Genbank Accession number NM_054039:

muFoxp3FWD (forward)

*Eco*RI Start
5' – GAGAGA**GAATTC**GCCACC**ATG**CCCAACCCTAGGCCAGCCAAG –3'

muFoxp3REV (reverse)

Stop
5' – GAGAGA**GCGGCCGCTCA**AGGGCAGGGATTGGAGCACTTG –3'
*Not*I

2.2 Cell Culture Methods

2.2.1 Medium for mammalian cells

All human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI 1640 medium supplemented with 5% (v/v) human AB serum, 2mM L-glutamine, 50U/mL penicillin and 50μg/mL streptomycin. Mouse splenocyte-derived cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 50U/mL penicillin and 50μg/mL streptomycin. CHO-K1 cells were maintained in DMEM medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 50U/mL penicillin, 50μg/mL streptomycin and 1X non-essential amino acids. CHO-K1 cells are adherent cells and were removed by the addition of trypsin-EDTA.

2.2.2 Isolation of human PBMCs from blood

Human peripheral blood was obtained from normal healthy donors and was given with informed consent. Whole blood was taken by venous puncture and collected into heparinised containers (BD Biosciences). Blood was diluted 1:1 using RPMI 1640 medium, prior to layering 30mL of diluted blood over 20mL of Ficoll-Paque^{TM} PLUS (Amersham Biosciences). Layered blood was separated by density gradient centrifugation (470g for 25 minutes, no brake). The PBMC interface was pipetted off into fresh human AB serum-containing medium and centrifuged at 470g for a further 10 minutes. The cell pellet was resuspended in fresh medium and centrifuged at 210g for 10 minutes to remove contaminating platelets. This was followed by a final wash and centrifugation step at 300g for 5 minutes.

2.2.3 Isolation of human CD4+ and CD8+ T cells from peripheral blood

Human PBMCs were isolated from peripheral blood by density gradient centrifugation as described above. The CD4+ T cells and CD8+ T cells were purified *via* negative selection using MACS human CD4+ and CD8+ T cell negative selection isolation kits, LS+ separation columns and a Quadromacs magnetic separator, according to the manufacturer's instructions (Miltenyi Biotec, Bergish Gladbach, Germany.). The negative selection kits allow the isolation of unlabelled CD4+ or CD8+ T cells by depletion of non-CD4+ or non-CD8+ T cells with a cocktail of biotin-conjugated antibodies to CD14, CD16, CD19, CD36, CD56, CD123, TCR$\gamma\delta$ and glycophorin A. The addition of anti-biotin coated microbeads ensures the antibody bound cells are retained on the LS+ separation column, placed within a magnetic field, whilst the unbound cells are collected as flow-through. Where

required, the LS+ column was removed from the magnetic field and the bound cells were eluted and used as CD4-depleted PBMCs.

2.2.4 Isolation of human CD4+CD25+ and CD4+CD25- T cells from peripheral blood

Purified human CD4+ T cells were further separated into CD25+ and CD25- fractions using MACS human CD25 microbeads (Miltenyi Biotec). Purified CD4+ T cells were resuspended in 95μL of MACS buffer per 10^7 cells. 5μL of CD25 microbeads per 10^7 cells were added and the cells were incubated for 10 minutes at 4°C. The cells were washed in 10-20 times the original volume with MACS buffer and resuspended in 500μL of MACS buffer per 10^8 total cells. A LS+ column was placed into the QuadroMacs magnetic separator and prewashed with 3mL of MACS buffer. Cell suspension was loaded onto the column and the eluant was collected. Four further washes with 3mL of MACS buffer were conducted and the additional eluant was collected to ensure all the CD25- cells were isolated. The column was removed from the magnet and the bound cells were eluted in 5mL of MACS buffer (CD25+ fraction). Eluted cells were reapplied to a second LS+ column and the procedure was repeated to ensure optimal enrichment of the CD25+ population. The flow-through was discarded at this stage.

2.2.5 Isolation of human CD4+CD95+ and CD4+TNFR2+ T cells from peripheral blood

CD4+CD95+ and CD4+TNFR2+ T cells were isolated using a MACS two-step positive selection method with an anti-CD95-PE or anti-TNFR2-PE antibody and anti-PE-MACS beads (Miltenyi Biotec). Briefly the purified CD4+ T cells were

resuspended in 100μL of MACS buffer per 10^7 cells. 10μL of CD95-PE or TNFR2-PE antibody was added per 10^7 cells and the cells were incubated for 10 minutes at 4°C. Cells were washed in 10-20 times the original volume with MACS buffer and resuspended in 90μL of MACS buffer per 10^7 cells. 10μL of anti-PE microbeads (Miltenyi Biotec) were added per 10^7 cells and the cells were incubated for a further 10 minutes at 4°C. Cells were washed as above and resuspended in 500μL of MACS buffer per 10^8 total cells. Cells were purified into CD95+ and CD95- fractions (or TNFR2+ and TNFR2- fractions) following the method described for the purification of human CD4+CD25+ and CD4+CD25- T cells.

2.2.6 Isolation of mouse CD4+CD25+ and CD4+CD25- splenocytes

Spleens were obtained from 6-8-week-old BALB/c mice. Single cell suspensions were prepared by mechanical disaggregation through sterile nylon gauze (40μm). Contaminating erythrocytes were removed by a 5 minute lysis step in red cell lysis buffer. Cells were washed twice in RPMI 1640 medium containing 10% FCS and the CD4+ T cells were purified *via* negative selection using MACS mouse CD4+ T cell isolation kits, according to the manufacturer's instructions (Miltenyi Biotec). Where required, the LS+ column was removed from the magnetic field and the bound cells were eluted and used as CD4-depleted splenocytes. CD4+CD25+ T cells were isolated using a MACS two-step positive selection method with an anti-CD25 PE antibody and anti-PE-MACS beads (Miltenyi Biotec) as described for the purification of human CD4+CD95+ and CD4+CD95- T cells.

2.2.7 Human CD4+CD25+ suppression assays in the presence of PHA-L

CD4+ T cell-depleted PBMCs were γ-irradiated for 50 minutes (25 sieverts, ^{137}Cs source). The irradiated CD4-depleted PBMCs ($5x10^4$/well) were cultured in 96-well U-bottomed plates with $2.5x10^4$/well purified CD4+CD25- or CD4+CD25+ T cells or both ($2.5x10^4$ each per well) in the presence of 1μg/mL PHA-L for 3 days at 37°C/ 5% CO_2. On day 3, 0.5μCi/well ^{3}H-thymidine (Amersham Biosciences) was added for 4-6 hours. Cells were then harvested onto glass-fibre filter mats (Perkin Elmer, Beaconsfield, U.K.) using a Skatron cell harvester and radionuclide uptake was measured by liquid scintillation counting. Supernatants were removed from duplicate cultures and assayed for IL-2, TNFα, IL-5 and IFNγ. In some experiments, antibodies and exogenous cytokines (IL-2, TNFα and lymphotoxin (LT)) were added to the cultures at the start of the experiment. All antibodies were added at 10μg/mL unless otherwise indicated and those used are summarised in table 2.1. Exogenous cytokines were added at the concentrations indicated.

2.2.8 Human CD4+CD25+ suppression assays in the presence of tetanus toxoid (TT)

Irradiated CD4+ T cell-depleted PBMCs ($1x10^5$/well) were cultured in 96-well U-bottomed plates with $5x10^4$/well purified CD4+CD25- or CD4+CD25+ T cells or both ($5x10^4$ each per well) in the presence of 1μg/mL TT for 7 days at 37°C/ 5% CO_2. On day 7, 0.5μCi/well ^{3}H-thymidine was added for 4-6 hours. Cells were then harvested onto glass-fibre filter mats using a Skatron cell harvester and radionuclide uptake was measured by liquid scintillation counting. Supernatants were removed from duplicate cultures and assayed for IL-2 and IFNγ. In some experiments exogenous IL-6 was added to the cultures at the concentrations indicated.

2.2.9 Cytokine assays.

The commercially available ELISA Duoset kits for human cytokines IL-2, IL-10, IFNγ and TNFα (R&D Systems, Abingdon, U.K.) were used as indicated by the manufacturer. All standard curves were constructed and data analysed using Genesis II software (Labsystems, Altrincham, UK). Where indicated, additional measurements of the human cytokines IL-5, TNFα and IFNγ were carried out using Luminex technology from R&D Systems, according to the manufacturer's instructions.

2.2.10 Mouse CD4+CD25+ suppression assays

CD4+ T cell-depleted splenocytes were γ-irradiated for 50 minutes (25 sieverts, ^{137}Cs source). Irradiated CD4+ T cell-depleted splenocytes ($5x10^4$/well) were cultured in 96-well U-bottomed plates with $1x10^4$/well purified CD4+CD25- or CD4+CD25+ T cells or both ($1x10^4$ each per well) in the presence of 1μg/mL anti-CD3 antibody for 5 days at 37°C/ 5% CO_2. On day 5, 0.5μCi/well ^3H-thymidine was added for 4-6 hours. Cells were then harvested onto glass-fibre filter mats using a Skatron cell harvester and radionuclide uptake was measured by liquid scintillation counting. Some experiments were performed in the presence of exogenous mouse IL-2 at the concentrations indicated.

2.2.11 Flow cytometric analysis

Round-bottomed 5mL polypropylene Falcon tubes were used for flow cytometric analysis (BD Biosciences). Cells ($5x10^4$-$1x10^5$/tube) were washed in 3mL/tube of ice-cold PBS supplemented with 5% (v/v) FCS and 0.1% (w/v) sodium azide (FACS buffer) and then incubated with the appropriately diluted antibody (or antibodies) for 30 minutes on ice. The cells were then washed twice in 3mL/tube of FACS buffer and

resuspended in 200μL/tube of FACS buffer. Analysis of the cells was carried out on a FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA). All antibodies used are listed in table 2.2. CD33-FITC and CD33-Alexa 488, both purified and conjugated in-house, were used at a final concentration of 1μg/mL. Propidium iodide (PI) (BD Pharmingen) was used at a final concentration of 0.5μg/mL.

2.2.12 Intracellular staining

Intracellular staining was carried out using Immunotech's IntraPrep™ permeabilisation reagent, according to the manufacturer's instructions (Beckman Coulter, High Wycombe, U.K.).

2.3 Protein Methods

2.3.1 Preparation of cell lysates

Typically $5x10^5$-$1x10^6$ cells were washed twice in PBS to remove all traces of serum. Pelleted cells were resuspended in 20μl of ice-cold lysis buffer (9mL of membrane preparation buffer, 1mL of 10% Triton X-100 and a 'complete' protease inhibitor tablet (Roche Molecular Biochemicals, Lewes, U.K.). Cells were incubated on ice for 5 minutes. Lysed cells were centrifuged at 16,100g for 10 minutes at 4°C in a bench-top centrifuge and supernatants were removed and stored at –20°C awaiting further analysis.

2.3.2 Determination of protein concentration

The total protein concentration of the cell lysates was determined using Micro BCA

Protein Assay Reagents (Pierce Biotechnology, Rockford, U.S.A.) in accordance with the manufacturer's protocol. Cell lysis buffer was used as the diluent for BSA standards and samples.

2.3.3 Immunological detection of protein (Western Blotting)

Samples were prepared for Western blot analysis by the additional of 6X protein sample buffer. Typically 3μL of buffer was added to a 15μL sample. Samples were incubated at 100°C for 5 minutes and then briefly centrifuged in a bench-top centrifuge for 10 seconds. Samples were loaded onto 10% (w/v) Tris-Glycine pre-cast polyacrylamide gels (Invitrogen) alongside prestained molecular weight markers. Gels were electrophoresed in 1X running buffer at 100V/35mA for approximately 90 minutes, until the dye front had reached the bottom of the gel. Proteins were transferred to Immobilon-P PVDF membrane, which had been pre-wetted with methanol. Overnight transfer was carried out in transfer buffer at 80mA using a Hoefer TE series Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, U.S.A.). The blot was blocked in PBS containing 2% (w/v) milk powder for a minimum of 1 hour and then probed for 1-3 hours at room temperature with polyclonal rabbit antisera raised against recombinant human FOXP3, diluted 1:1,000 in PBS containing 1% (w/v) milk powder and 0.1% (v/v) Tween-20. The blot was thoroughly washed in PBS containing 0.1% (v/v) Tween-20 before incubation for 1 hour at room temperature with goat anti-rabbit Fc-HRP (Stratech Sci. Ltd, Luton, U.K.) diluted 1:10,000 in PBS containing 1% (w/v) milk powder and 0.1% (v/v) Tween-20. After a further thorough washing step, the blot was developed using Pierce Biotechnology's Supersignal reagents (Illinois, U.S.A.) in accordance with the manufacturer's instructions. The signal was detected using x-ray film, which was

processed using an X-Omat automatic developer (Kodak Ltd., Hemel Hempstead, U.K.)

2.4 DNA Methods

2.4.1 PCR amplification of DNA fragments

Amplification of both human *FOXP3* and mouse *Foxp3* was performed using Novagen's KOD Hot Start DNA Polymerase kit (Merck Biosciences, Nottingham, U.K.). To optimise PCR conditions, initial PCR reactions were carried out in a total volume of 20μL which contained approximately 100ng of cDNA, 0.3μM each oligonucleotide, 0.2mM dNTP mix (dATP, dCTP, dGTP, dTTP), 2μL 10X PCR buffer, 1mM $MgSO_4$, 0.4μL KOD polymerase, 1-5% sterile 20% DMSO (1, 2, 3, 4 or 5μL) and sterile dH_2O.

When PCR reactions were performed to generate DNA for cloning, reactions were scaled up to 100μL, with each component in the same proportion as described above for the 20μL reactions. 5% DMSO was used in these reactions. Oligonucleotides were designed to have a melting temperature (T_m) of at least 72°C. Reactions were cycled under the following conditions:

94°C	3 minutes	
94°C	30 seconds	35 cycles
60°C	30 seconds	
68°C	2 minutes	
68°C	5 minutes	
4°C	constant	

Post-reaction, samples were analysed by agarose gel electrophoresis.

2.4.2 Analysis of DNA fragments by agarose gel electrophoresis

DNA samples were analysed by horizontal gel electrophoresis at 80V using 1% (w/v) agarose gels in 1xTAE buffer. Appropriate size markers were included on each gel. Ethidium bromide was added to the gel to give a final concentration of 0.5μg/mL. DNA bands were visualised using ultraviolet (uv) transillumination at 254nm. Where agarose gel electrophoresis was used for the purification of DNA fragments, uv transillumination was at 366nm. This was to minimise the chance of uv-induced mutations.

2.4.3 Phenol-chloroform extraction of DNA

After some PCR reactions and all restriction enzyme digests prior to purification by gel electrophoresis, DNA was extracted with phenol/chloroform/isoamyl alcohol (IAA). Briefly, to 100μL of PCR reaction mix, 750μL phenol/chloroform/iosamyl alcohol, 80μL TE buffer and 20μL of HI TNE buffer were added and mixed well. Centrifugation (16,100g for 2 minutes) was used to separate the aqueous and organic layers. Aqueous layer was carefully removed and transferred to a fresh eppendorf tube. Any remaining phenol/chloroform/iosamyl alcohol was re-extracted with a further 200μL TE. Aqueous layer was combined with the first aqueous fraction and extracted with 750μL of chloroform and 15μL of IAA. DNA in the resultant aqueous layer was precipitated by the addition of 2.5 volumes of 100% ethanol and incubated at –20°C for 1 hour. DNA was collected by centrifugation at 16,100g for 10 minutes, washed once with 70% ethanol and finally resuspended in an appropriate volume of TE buffer.

2.4.4 Spin column purification of PCR products

Following phenol-chloroform extraction, the PCR products were subjected to a further purification step using Qiagen's QIAquick PCR purification kit, prior to restriction enzyme digestion. Purification of the mouse *Foxp3* PCR product was carried out using this method alone, without the initial phenol-chloroform step. Briefly 5 volumes of buffer PB were added to 1 volume of PCR reaction and mixed. The sample was loaded onto a QIAquick spin column and centrifuged at 16,100g for 1 minute; the flow-through was discarded. Column-bound DNA was washed with 750μL of buffer PE and centrifuged as before. The flow-through was discarded and the column was centrifuged for a further 1 minute. The DNA was eluted off the column by centrifugation following the addition of 50μL of buffer EB and an incubation step for 1 minute at room temperature.

2.4.5 Restriction enzyme digests

For sub-cloning into an expression plasmid, the PCR product (purified as described above, sections 2.4.3 and 2.4.4), or approximately 10μg of plasmid DNA, was incubated with 20 units of each restriction enzyme in the appropriate buffer, overnight at 37°C. One unit is defined as the amount of enzyme required to completely digest 1μg of DNA in 1 hour. The digested fragments were then subjected to a phenol-chloroform extraction (2.4.3) prior to purification by gel extraction. For diagnostic analysis to detect clones containing the correct sized insert fragments, 0.5-1μg of DNA was incubated with 5 units of each restriction enzyme in the appropriate buffer for 2-4 hours at 37°C. Digested plasmids were then analysed by gel electrophoresis.

2.4.6 Purification of DNA fragments by gel extraction.

DNA fragments were purified from 1% agarose gels using Qiagen's QIAquick Gel Extraction Kit, in accordance with the manufacturer's instructions. Briefly the desired band was excised from the agarose gel under low uv wavelength light (366nm). The fragment was weighed and 3 volumes of buffer QG were added (the assumption was made that 100mg of gel was equivalent to 100μL volume). Following an incubation step at 50°C for 10 minutes to melt the agarose, 1 volume of isopropanol was added and the sample was loaded onto a QIAquick spin column. The column was centrifuged at 16,100g for 1 minute and the flow-through was discarded. The column was washed with 750μL of buffer PE. Finally the DNA was eluted in 50μL of buffer EB.

2.4.7 Ligation of DNA fragments

Ligation of gel purified DNA fragments was performed in a total volume of 20μL, comprising 2μL 10X ligation buffer, 0.4μL T4 DNA ligase (HC), approximately 50ng of vector DNA and a molar equivalent of insert fragment. Control reactions containing no insert were also carried out to allow assessment of the efficiency of the transformation. Ligation reactions were incubated overnight at 16°C, prior to transformation into *E.coli* cells.

2.4.8 Transformation of DNA into competent E.coli cells

Sub-cloning grade XL-1 blue *E.coli* cells were used for all transformations (Stratagene, California, U.S.A.). 60μL of cells were gently thawed on ice and 1μL of β-mercaptoethanol was added. Cells were incubated on ice for 5 minutes then 1μL of ligation mix was added. Cells were incubated on ice for a further 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds and incubated for a further 2 minutes

on ice. 950μL of SOC medium was added and the cells were incubated on a shaker platform at 37°C for 60 minutes. An appropriate volume of bacterial culture (typically a range of volumes from 10-200μL were used) was then streaked out onto LB-agar plates supplemented with 100μg/mL carbenicillin. Plates were incubated at 37°C overnight.

2.4.9 Small-scale isolation of plasmid DNA.

Small-scale miniprep columns were used to prepare plasmid DNA for subsequent analysis by restriction enzyme digest or DNA sequencing. A single colony of transformed *E.coli* was used to inoculate 10mL of 2X TY culture broth containing 100μg/mL carbenicillin. Culture was grown overnight at 37°C with shaking. Bacterial pellet was collected by centrifugation (1,500g for 15 minutes). Plasmid DNA was extracted using Qiagen's miniprep columns, in accordance with the manufacturer's instructions.

2.4.10 Large-scale isolation of plasmid DNA.

Larger quantities of plasmid DNA were required for transfection experiments. A single colony of transformed *E.coli* was used to inoculate 10mL of 2X TY culture broth containing 100μg/mL carbenicillin. Culture was grown for 8 hours at 37°C with shaking before being used to inoculate 200mLs of 2X TY culture broth containing 100μg/mL carbenicillin. Following overnight culture at 37°C with shaking, the bacterial pellet was collected by centrifugation (4,600g for 15 minutes at 4°C). Plasmid DNA was then extracted in accordance with the manufacturer's instructions.

2.4.11 Optical density measurement of DNA

The concentration of DNA was determined *via* the measurement of the optical density at 260nm, using a quartz cuvette. An optical density reading of 1.0 was assumed to be equivalent to a concentration of 50μg/mL.

The concentration of DNA was hence calculated using the formula shown below:

$$(OD_{260} \times 50 \times \text{dilution factor})/ 1000 = \text{mg/mL}$$

The purity of DNA was also calculated by dividing the OD_{260} by the OD_{280}. Good levels of DNA purity give a value between 1.8-2.0.

2.4.12 DNA sequencing

DNA sequencing reactions were performed in 0.2mL thin-walled tubes (Anachem Ltd). Each reaction comprised 250ng of DNA, 5 pmoles sequencing primer, 1.5μL sequencing buffer and 1.5μL Big Dye Terminator Reaction Ready sequencing premix. Both the sequencing buffer and sequencing premix were included in the Big Dye Terminator Sequencing Ready Reaction DNA sequence kit, (Applied Biosystems). Distilled water was added to bring the volume to 7.5μL. The reaction was cycled under the following conditions:

96°C	30 seconds	
96°C	10 seconds	25 cycles
50°C	5 seconds	
60°C	4 minutes	
4°C	constant	

DNA was then precipitated by the addition of 0.75μL 3M sodium acetate (pH 4.6) and 19.23μL 95% ethanol to each tube. Samples were then incubated at room temperature for 15 minutes and centrifuged at 1,500g for 45 minutes at 4°C. The resultant pellets

were washed with 70% ethanol, air-dried and resuspended in 10μL formamide. The samples were then analysed on a 3100 automated DNA sequencer (Applied Biosystems).

2.5 Transfection Methods

2.5.1 Optimisation of transfection conditions

Transfections were carried out using the Amaxa Nucleofector™ technology (Amaxa Biosystems, Cologne, Germany.). Initial experiments were carried out using CHO cells. $2x10^6$ CHO cells were resuspended in 100μL of Nucleofector™ solution T. The cells were then mixed with 5μg of pTr.*FOXP3* plasmid DNA and transferred to a cuvette. The cuvette was subjected to electroporation using programme U-24 in the Nucleofector™ electroporator. Cells were transferred to fresh medium and incubated for 24 hour at 37°C/5% CO_2. The cells were then analysed for GFP expression levels by flow cytometry. Optimisation of the human T cell transfection conditions was carried out using increasing numbers of cells and DNA, resuspended in Nucleofector™ human T-cell solution. Cells were subjected to electroporation programme U-13.

2.5.2 Transfection of human CD4+ or CD8+ T cells with FOXP3 for functional studies

$4x10^6$ purified human CD4+ or CD8+ T-cells were resuspended in 100μL of Nucleofector™ human T-cell solution. The cells were then mixed with 3μg of chimeric receptor DNA and 9μg of plasmid DNA containing either full-length *FOXP3*, an isoform of *FOXP3,* FKH mutant, or plasmid DNA with no insert, and

transferred to a cuvette. The cuvette was subjected to electroporation using programme U-13 in the Nucleofector™ electroporator. Cells were immediately transferred to fresh culture medium and rested at 37°C for 4 hours. Following counting, cells were seeded at $5x10^5$ cells/well in either 96-well flat-bottomed CD33-coated plates (5μg/mL) or uncoated plates (stimulated versus unstimulated). The cells were then incubated for a further 24 hours. Supernatants were removed for cytokine analysis and cells were removed for analysis of cell viability, chimeric receptor expression levels and the levels of CD69 and CD25 expression, by flow cytometry. Cells were also harvested for analysis by Western blotting. For single transfections, 3μg of chimeric receptor and 9μg of *FOXP3* or control DNA (empty vector or FKH mutant) was used, per $4x10^6$ cells.

2.5.3 Fluorescence microscopy

Cells were co-transfected as described above and incubated for 24 hours without CD33 stimulation. A dead cell removal step was carried out using a dead cell removal kit (Miltenyi Biotec) according to the manufacturer's instructions prior to staining. Cytospins were prepared using $1x10^5$ cells/slide. The cells were fixed and permeabilised for 10 minutes in acetone and then air-dried for 30 minutes, before staining with 100μL/slide of pre-mixed CD33-FITC (1μg/mL) and rabbit anti-sera raised against human FOXP3 (1:100). Slides were incubated at room temperature for 30 minutes, before being washed in PBS supplemented with 5% FCS and 0.1% sodium azide and air-dried. Goat anti-rabbit IgG (Heavy+Light) Alexa Fluor 660 antibody (Molecular Probes, Leiden, The Netherlands.) was added to each slide at a working concentration of 10μg/mL and the slides were incubated for a further 30 minutes at room temperature. The slides were thoroughly washed in PBS and

analysed on a Leica DMRXA fluorescence microscope. Images were manipulated using Openlab 2.2.0 deconvolution software (Improvision, Coventry, U.K.).

2.5.4 *Proliferation assays with FOXP3-transfected cells.*

CD4+ T cell-depleted PBMCs were γ-irradiated for 50 minutes (25 sieverts, ^{137}Cs source). The irradiated CD4+ T cell-depleted PBMCs ($5x10^4$/well) were incubated in 96-well U-bottomed plates with $2.5x10^5$ *FOXP3* or control vector-transfected cells and 1μg/mL soluble anti-CD3. On day 3, 0.5μCi/well ^3H-thymidine was added for 4-6 hours. Cultures were harvested and radionuclide uptake measured by liquid scintillation counting.

2.5.5 Suppression assays with chimeric receptor-transfected CD4+ T cells.

CD4+ T cells transfected with CD28/TCRζ chimeric receptor alone ($2x10^5$/well) were co-cultured in a 1:2 ratio with CD4+ T cells co-transfected with either *FOXP3* and CD28/TCRζ chimeric receptor, or FKH mutant and CD28/TCRζ chimeric receptor ($4x10^5$/well) in 96-well flat-bottomed CD33-coated plates for 24 hours at 37°C. Supernatants were removed and assayed for IL-2 production. This was repeated using CD4+ T cells co-transfected with either *FOXP3* and TCRζ chimeric receptor, or FKH mutant and TCRζ chimeric receptor.

CD28/TCRζ-transfected cells ($1.5x10^4$/well) were also co-cultured in a 1:1 ratio with autologous preactivated CD4+CD25+ or CD4+CD25- T cells in 96-well flat-bottomed CD33-coated plates for 24 hours at 37°C. Supernatants were removed and assayed for IL-2 production. The preactivated cells were activated for 24 hours with 1μg/mL PHA-L in the presence of autologous CD4+ T cell-depleted PBMCs. The cells were

then repurified using a MACS CD4+ T cell negative isolation kit prior to addition to the suppression assay.

2.5.6 Suppression assays with chimeric receptor-transfected CD4+CD25+ regulatory T cells.

Human CD4+ T cells were purified from 200mL of peripheral blood. The purified CD4+ T cells were resuspended in Nucleofector™ human T-cell solution to give a cell concentration of $4x10^6$cells/100μL solution. Each 100μL of cell suspension was then mixed with 3μg of CD28/TCRζ chimeric receptor DNA and transferred to a cuvette. The cuvette was subjected to electroporation using programme U-13 in the Nucleofector™ electroporator. Cells were immediately transferred to fresh culture medium and rested at 37°C for 4 hours. The cells were then purified into CD25+ and CD25- fractions following the protocol detailed in section 2.2.4. Chimeric receptor transfected CD4+CD25+ regulatory T cells ($5x10^4$/well) were co-cultured in 96-well flat-bottomed CD33-coated plates with $2.5x10^4$/well chimeric receptor transfected CD4+CD25- T cells (2:1 ratio). Cells were incubated for a further 24 hours at 37°C/5% CO_2. Supernatants were then removed and assayed for IL-2 production. Control wells included chimeric receptor transfected CD4+CD25+ regulatory T cells ($5x10^4$/well) alone and chimeric receptor transfected CD4+CD25- T cells alone at $2.5x10^4$/well, $5x10^4$/well and $7.5x10^4$/well. Both populations of transfected cells were also cultured for 24 hours at 37°C/5% CO_2 in 96-well flat-bottomed uncoated plates ($5x10^4$/well). Cells were then removed and viabilities and chimeric receptor expression levels were determined by flow cytometry.

Chapter 3

Characterisation of Human CD4+CD25+ Regulatory T cells

3.1 Introduction

The immune system is a highly complex entity requiring tightly controlled regulation to ensure tolerance to certain antigens and to prevent host tissue damage. Mechanisms that down-regulate an immune response are equally as important as mechanisms that activate a response. A seminal paper published in 1995, described a sub-population of CD4+ T cells whose function appeared to be the maintenance of self-tolerance in the periphery *via* down-regulation of the immune response (Sakaguchi *et al.*, 1995). These cells were characterised by the cell surface expression of CD25 and such was the impact of these studies, that the field of regulatory T cell biology was reborn. A plethora of papers have since been published on the phenotype of these naturally occurring CD4+CD25+ regulatory T cells, the activation requirements of these cells and the mechanisms by which these cells regulate. However, there are still many issues to be resolved before the biology of these cells is fully understood.

It is generally accepted that CD4+CD25+ regulatory T cells are derived from the thymus, although there may be mechanisms for generation of these cells in the periphery as well (Fehervari and Sakaguchi, 2004). They represent 5-10% of all peripheral CD4+ T cells and are characterised as being hyporesponsive *in vitro* to activation through the TCR (Jonuleit and Schmitt, 2003). In order to suppress CD4+CD25- responder cells, CD4+CD25+ regulatory cells require activation through the TCR and it appears that cell-cell contact is paramount for suppression *in vitro*. However, *in vivo* data suggests an additional role for soluble factors like IL-10 and TGFβ (von Boehmer, 2005). The ability to suppress responses to a wide range of

stimuli including self antigen, foreign antigen, dietary antigen and allo antigen, is an additional feature of these cells (Taams *et al.*, 2002).

Reliable cell surface biomarkers that identify CD4+CD25+ regulatory T cells specifically, is an area of much research. CD25 has proved to be a reliable marker for the identification of resting CD4+CD25+ regulatory T cells, however it also a marker of activated T cells. Some of the other cell surface markers that have been identified by various groups as markers for CD4+CD25+ regulatory T cells include CD103, OX40, 4-1BB, GITR, CTLA-4, TNFR2 and CD62L (Piccirillo and Thornton, 2004). Whether any of these markers also play a role in the functionality of these cells is unclear, as all appear to be dispensable for suppressor function.

The aim of this chapter was to address some of these issues by attempting to more fully characterise human CD4+CD25+ regulatory T cells, both in terms of cell surface marker expression and functionality. The expression of surface markers on both freshly isolated and three day activated CD4+CD25+ regulatory T cells was investigated and compared to CD4+CD25- T cells. Functional assays were also developed using both a polyclonal stimulus (PHA-L) and an antigen specific stimulus (tetanus toxoid). The mechanism of suppression was investigated by the use of antibodies to molecules implicated in regulatory T cell function, including antibodies to CTLA-4, IL-10, TGFβ, CD25 and IL-2. The effect of certain cytokines on the ability of CD4+CD25+ regulatory T cells to regulate was also studied. Lastly, the isolation of regulatory CD4+ T cells on the basis of TNFR2 or CD95 expression and the subsequent ability of these cells to regulate was evaluated.

3.2 Results

3.2.1 Purification of human CD4+CD25+ T cells from peripheral blood

Human CD4+CD25+ T cells were purified from PBMCs using a two-step MACS magnetic bead system. Initially the CD4+ T cells were isolated by negative selection followed by a positive selection for CD25. Analysis by flow cytometry of the starting population of PBMCs and the purified CD4+CD25+ and CD4+CD25- T cell populations, are shown in figure 3.1.

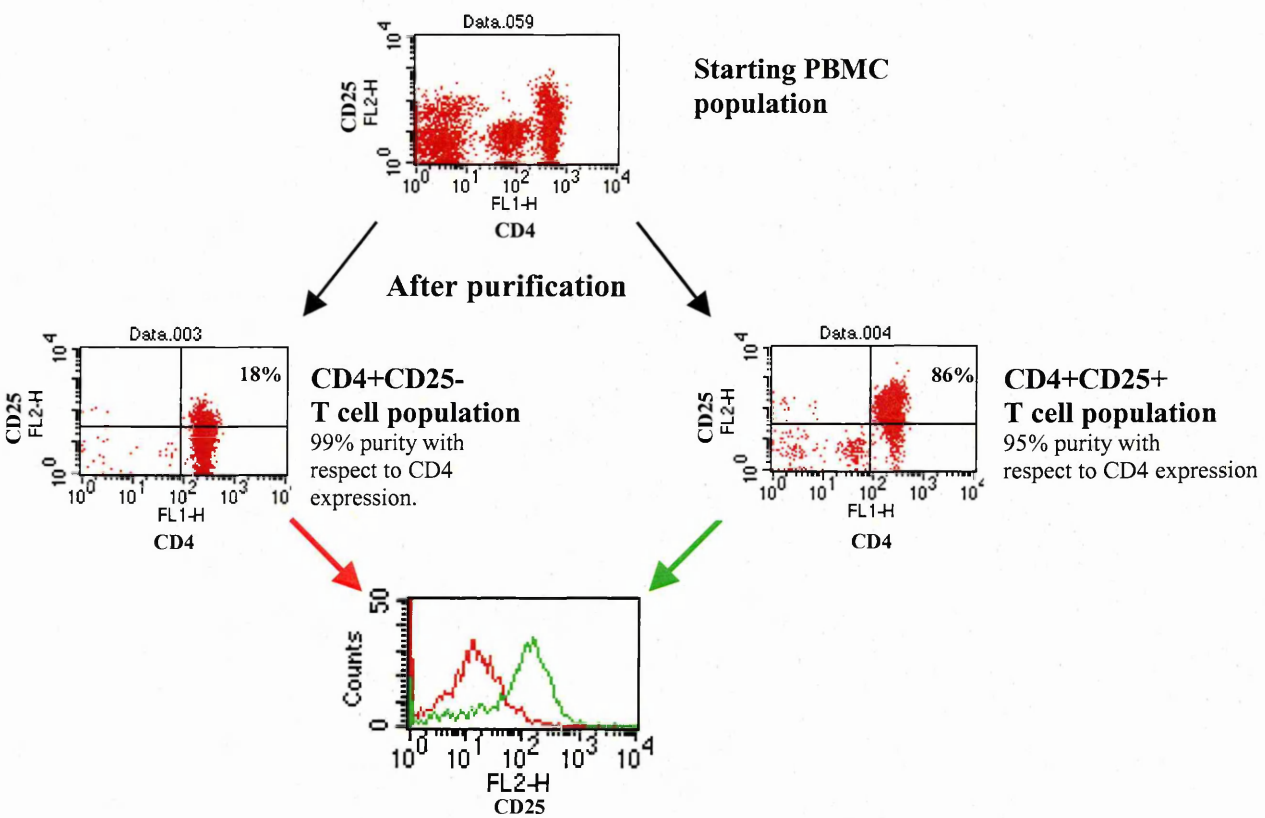


Figure 3.1 Isolation of human CD4+CD25+ regulatory T cells from peripheral blood. CD4+ T cells were isolated from PBMCs using a MACS CD4+ negative selection isolation kit and separated into CD25+ and CD25- fractions using MACS CD25 microbeads. The starting PBMC population and the purified CD4+ T cells were analysed by flow cytometry for CD25 expression. Gates for CD25 expression were set using a mouse IgG1-PE antibody control. Percentage values for the number of CD25 positive cells are displayed. Data shown is representative of one of numerous donors.

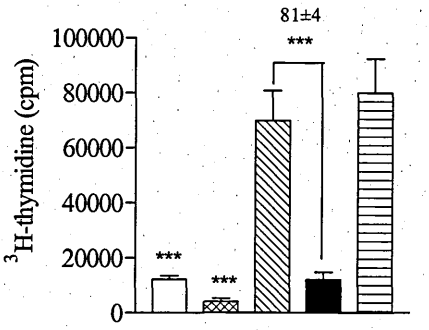
As is illustrated in the starting PBMC population in figure 3.1, there are 3 different cell populations staining for CD4 expression. The $CD4^{hi}$ population represent the CD4+ T cells whilst the population staining medium-low for CD4 expression represent monocytes. The CD4 negative population primarily encompasses CD8+ T cells and B cells. The monocyte population is removed during the CD4+ T cell negative selection isolation step. Following purification with CD25 microbeads, a good enrichment of CD25 positive cells was obtained with high levels of CD25 being expressed on 86% of the CD4+ T cells. The dot-plots show that the purity of the CD4+CD25- population was higher than the CD4+CD25+ population, in this example 99% versus 95% with respect to CD4 expression. Attempts to identify the contaminating population within the CD4+CD25+ population proved somewhat inconclusive and indeed, the levels of contaminating cells differed across donors. However the purity of the CD4+CD25+ population was consistently greater than 90%.

3.2.2 Development of functional CD4+CD25+ suppressor assays

Having isolated a population of CD4+ T cells enriched for CD25 expression, the next step was to establish whether the cells were capable of inhibiting proliferation of CD4-CD25- responder T cells in a functional assay. Purified CD4+CD25+ T cells were co-cultured with purified CD4+CD25- cells in the presence of irradiated CD4+ depleted PBMCs and PHA-L. As is illustrated in figure 3.2a, the purified CD4+CD25+ cells significantly suppressed the proliferative response of the CD4+CD25- cells in response to PHA-L. Statistical analysis was by ANOVA and this analysis allows for comparison between multiple groups, however significance is

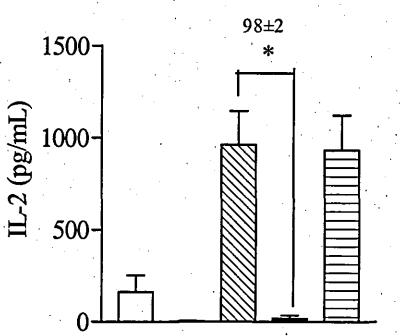
usually only reached with experimental numbers greater than 3. As the majority of the experiments described in this chapter have experimental numbers of 3, it was decided that a Student T-test would be sufficient for statistical analysis of the remaining data described in this chapter. Hence, the levels of IL-2 (figure 3.2b), TNFα (figure 3.2c), IL-5 (figure 3.2d) and IFNγ (figure 3.2e) were also significantly inhibited, as determined by a Student T-test. The CD4+CD25+ regulatory T cells also suppressed a cell population from undergoing low levels of proliferation and producing cytokines within the irradiated CD4-depleted cell population. This population although not formally identified, is thought to consist primarily of CD8+ T cells, as PHA-L is a potent T cell stimulator. For clarification, the abbreviation '++' depicted on the graphs refers to double the number of CD4+CD25- T cells. This is to control for the total number of cells per well.

PHA-L is a mitogen and acts by cross-linking glycoproteins and glycolipids on the cell surface resulting in cellular activation. However it is not a physiological means of activating cells. As an alternative, antigen-driven assays were developed using tetanus toxoid. Purified CD4+CD25+ T cells were co-cultured with purified CD4+CD25- cells in the presence of irradiated CD4-depleted PBMCs and tetanus toxoid. The data illustrated in figure 3.3a shows that the CD4+CD25+ cells significantly inhibited the proliferative response of the CD4+CD25- cells in response to stimulation with tetanus toxoid. Production of IL-2 and IFNγ was also inhibited by 59 ± 3% and 98 ± 2% respectively (figures 3.3b and 3.3c). These data confirm that peripheral blood-derived human CD4+CD25+ regulatory cells are capable of significantly inhibiting both the proliferative response of CD4+CD25-responder T cells and the release of both Th1 and Th2 cytokines, following either mitogenic or antigen-specific T cell stimulation.



irradiated CD4-depleted PBMCs	+	+	+	+	+
PHA-L	+	+	+	+	+
CD4+CD25+	-	+	-	+	-
CD4+CD25-	-	-	+	+	++

Figure 3.2a Inhibition of proliferation of CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 6 donors. Value above *** indicates percentage inhibition ± SEM. Statistical analysis was by ANOVA with Bonferroni post-test. ***, $p<0.001$ versus CD4+CD25- (bar3) and 2x CD4+CD25- (bar 5).



irradiated CD4-depleted PBMCs	+	+	+	+	+
PHA-L	+	+	+	+	+
CD4+CD25+	-	+	-	+	-
CD4+CD25-	-	-	+	+	++

Figure 3.2b Inhibition of IL-2 production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 3 and assayed for IL-2 levels by ELISA. Data shown are mean ± SEM of 3 donors.Value above * indicates percentage inhibition ± SEM. *,$p<0.05$, paired t-test.

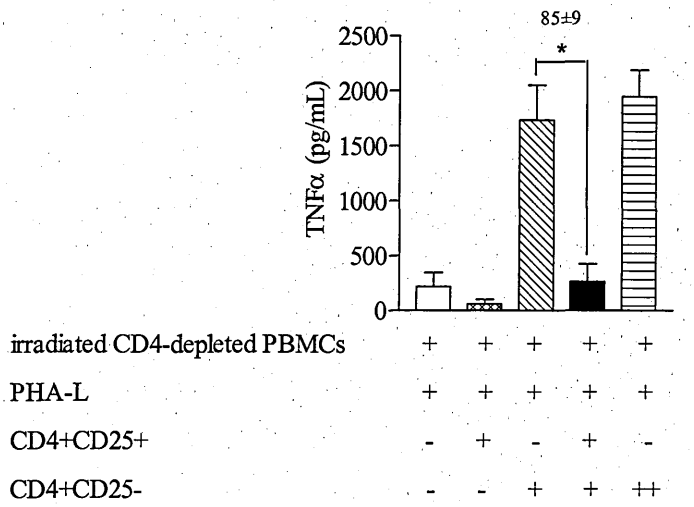


Figure 3.2c Inhibition of TNFα production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 3 and assayed for TNFα levels by LUMINEX. Data shown are mean ± SEM of 3 donors. Value above * indicates percentage inhibition ±SEM. *, $p<0.05$, paired t-test.

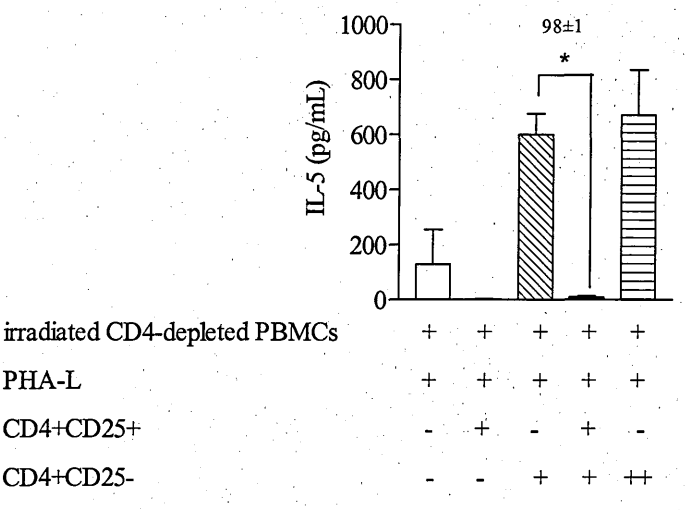


Figure 3.2d Inhibition of IL-5 production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 3 and assayed for IL-5 levels by LUMINEX. Data shown are mean ± SEM of 3 donors. Value above * indicates percentage inhibition ± SEM.*, $p<0.05$, paired t-test.

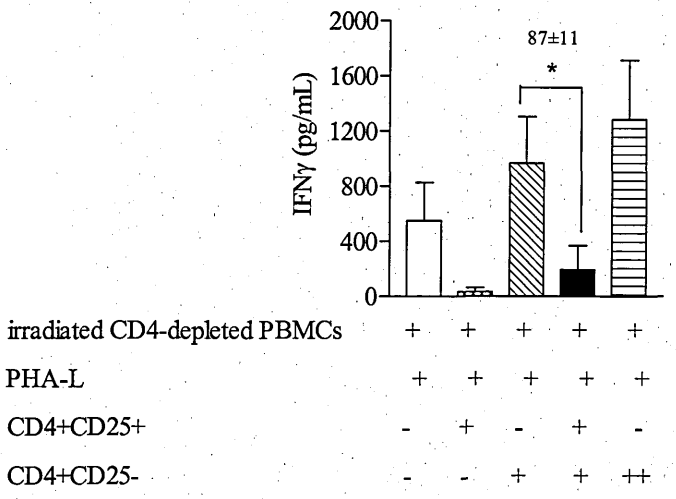


Figure 3.2e Inhibition of IFNγ production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 3 and assayed for IFNγ levels by LUMINEX. Data shown are mean ± SEM of 3 donors. Value above * indicates percentage inhibition ± SEM.*, $p<0.05$, paired t-test.

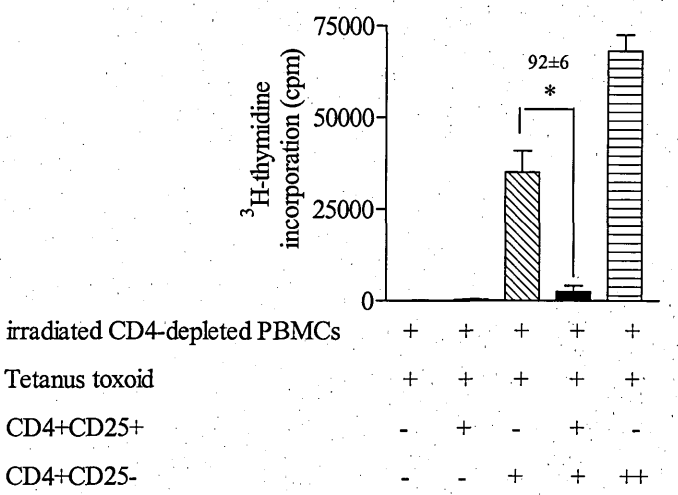


Figure 3.3a Inhibition of proliferation of CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with tetanus toxoid.
Purified CD4+CD25- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 7-day culture period. Data shown are mean ± SEM of 3 donors. Value above * indicates percentage inhibition ± SEM. *, $p<0.05$, paired t-test.

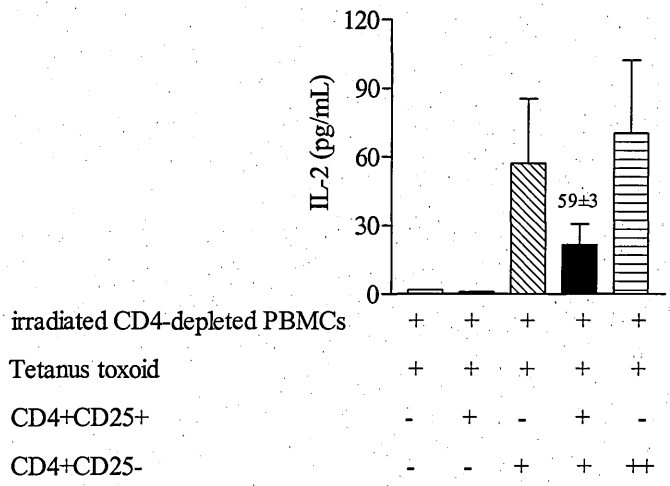


Figure 3.3b Inhibition of IL-2 production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with tetanus toxoid.
Purified CD4+CD25- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 7 and assayed for IL-2 levels by ELISA. Data shown are mean ± SEM of 3 donors. Value above bar indicates percentage inhibition ± SEM.

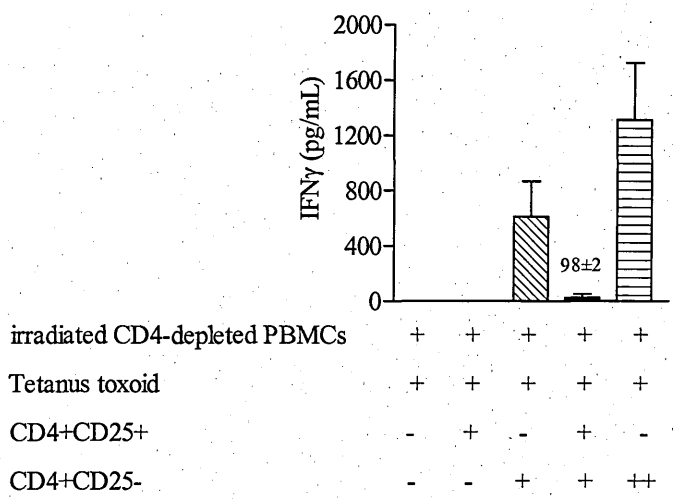


Figure 3.3c Inhibition of IFNγ production from CD4+CD25- responder cells by CD4+CD25+ regulatory cells following stimulation with tetanus toxoid.
Purified CD4+CD25- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Supernatant was removed on day 7 and assayed for IFNγ levels by ELISA. Data shown are mean ± SEM of 4 donors. Value above bar indicates percentage inhibition ± SEM.

3.2.3 Potency of the CD4+CD25+ regulatory T cell

To determine the potency of the CD4+CD25+ regulatory cells being isolated, a cell titration assay was established. The data illustrated in figure 3.4 shows that substantial inhibition of CD4+CD25- responder cells was achieved at a 1:1 cell ratio, where levels of 87% suppression were achieved. However even at a 3:1 ratio of responder to suppressor cells, the CD4+CD25+ were capable of approximately 50% inhibition. At a ratio of ten responders to one suppressor the levels of inhibition were negligible.

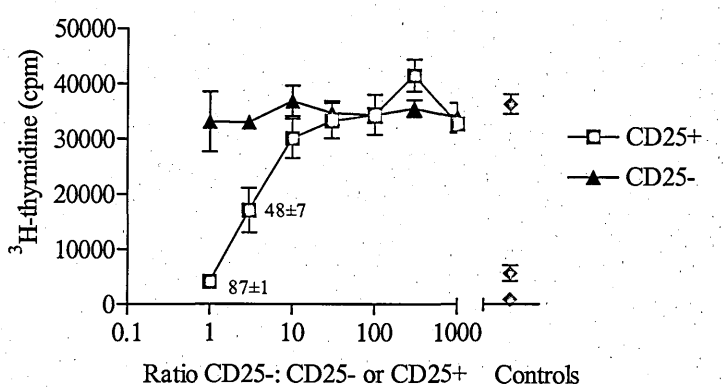


Figure 3.4 Titrating the suppressor response of CD4+CD25+ T cells.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of titrating numbers of CD4+CD25- or CD4+CD25+ T cells. ◇ represents irradiated CD4-depleted PBMCS alone. ◇ represents the addition of CD4+CD25+ cells. ◇ represents the addition of CD4+CD25- cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown is representative of one of three experiments. Error bars represent SD of triplicate wells. Values next to data points indicate percentage inhibition ± SD.

3.2.4 Investigation into whether IL-2 or certain proinflammatory cytokines can reverse CD4+CD25+-mediated suppression

The mechanism of suppression by human CD4+CD25+ regulatory cells *in vitro* is believed to be cell contact dependent. However there is conflicting data on the ability of different reagents to reverse suppression. In an attempt to determine whether the cells isolated in this study were responsive to IL-2, assays were established whereby increasing concentrations of human IL-2 was added to the co-cultures. Figure 3.5 clearly illustrates that in this study, the ability of CD4+CD25+ regulatory cells to suppress CD4+CD25- responder cells, was not reversed following the addition of exogenous IL-2.

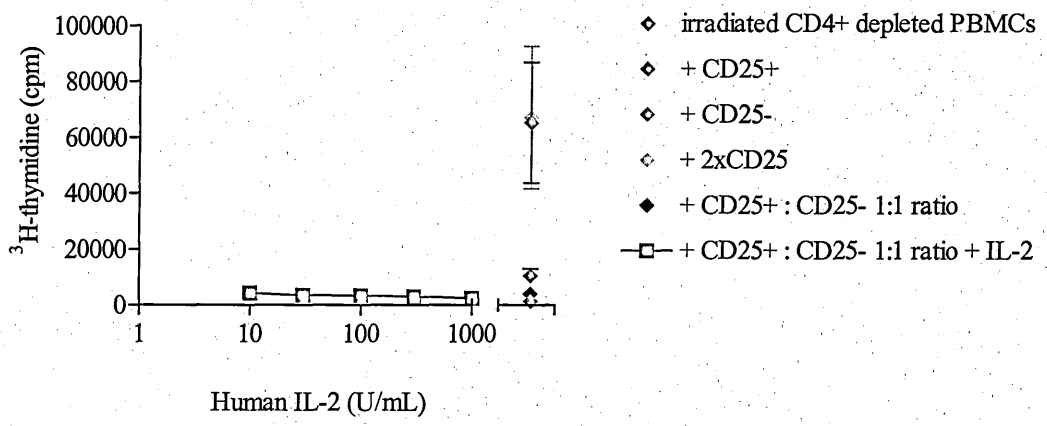


Figure 3.5 Investigating the ability of exogenous IL-2 to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ at a 1:1 ratio of T cells. Exogenous human IL-2 was added at the start of the assay at the concentrations indicated. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors.

To confirm that this result was not due to the nature of the activation stimulus, the experiment was repeated using tetanus toxoid as the stimulus (figure 3.6). A full dose response curve could not be set-up due to the increased numbers of cells required for

this assay. However, in this system also, the addition of exogenous IL-2 did not result in full reversal of CD4+CD25+ T-cell mediated suppression. Previous studies using this batch of IL-2 in T cell expansion cultures had confirmed its bioactivity at 50U/mL.

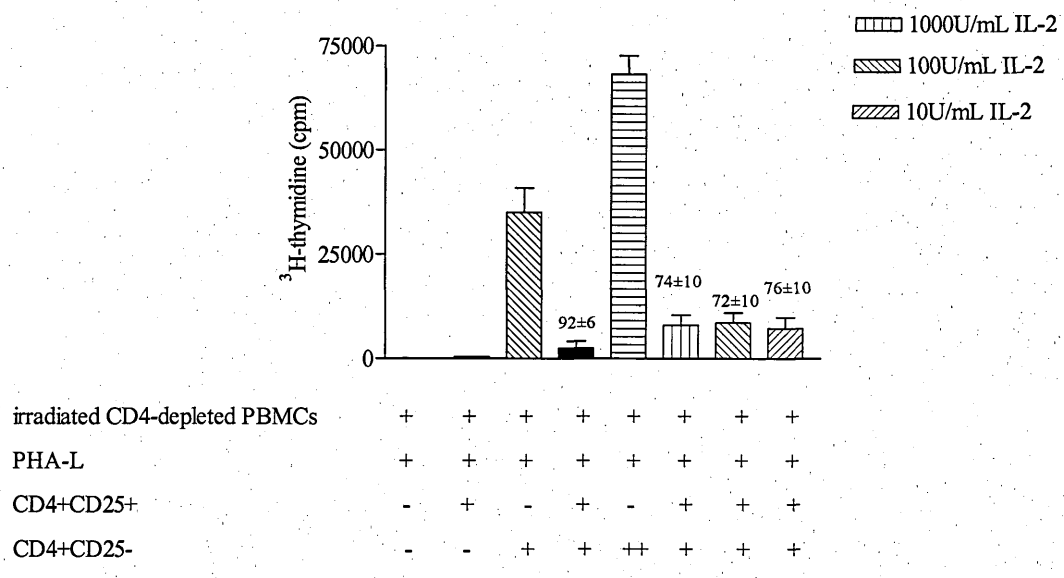


Figure 3.6 Investigating the ability of exogenous IL-2 to reverse CD4+CD25+ T cell mediated suppression following activation with tetanus toxoid.
Purified CD4+CD25- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Exogenous human IL-2 was added at the start of the assay at the concentrations indicated. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 7-day culture period. Values above bars indicate percentage inhibition ± SEM. Data shown are mean ± SEM of 3 donors.

In a highly pro-inflammatory environment, one might expect suppression to be overcome in order to allow the host to mount an effective immune response to the invading pathogen. In this system, IL-2 alone did not appear to be having a profound effect, however other more proinflammatory cytokines could play such a role. Thus experiments were set up investigating the effect of exogenous IL-6, TNFα and Lymphotoxin (LT), on the ability of CD4+CD25+ T cells to suppress CD4+CD25- responder cells. The data from these experiments is illustrated in figures 3.7a-b and clearly shows that none of the aforementioned cytokines appeared to affect the ability

of CD4+CD25+ cells to inhibit the proliferation of CD4+CD25- cells. The bioactivity of each of the cytokines had previously been confirmed in other cell-based assays.

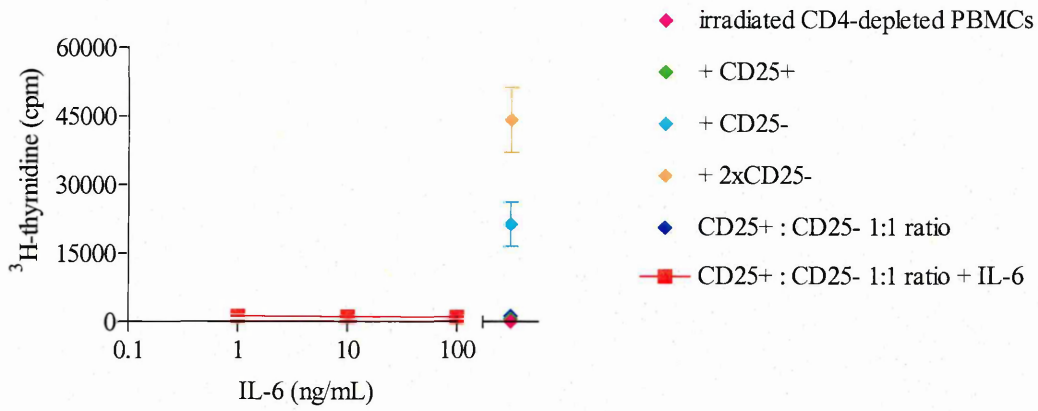


Figure 3.7a Investigating the ability of exogenous IL-6 to reverse CD4+CD25+ T cell mediated suppression following activation with tetanus toxoid.
Purified CD4+CD25- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Exogenous human IL-6 was added at the start of the assay at the concentrations indicated. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 7-day culture period. Data shown are mean ± SEM of 3 donors.

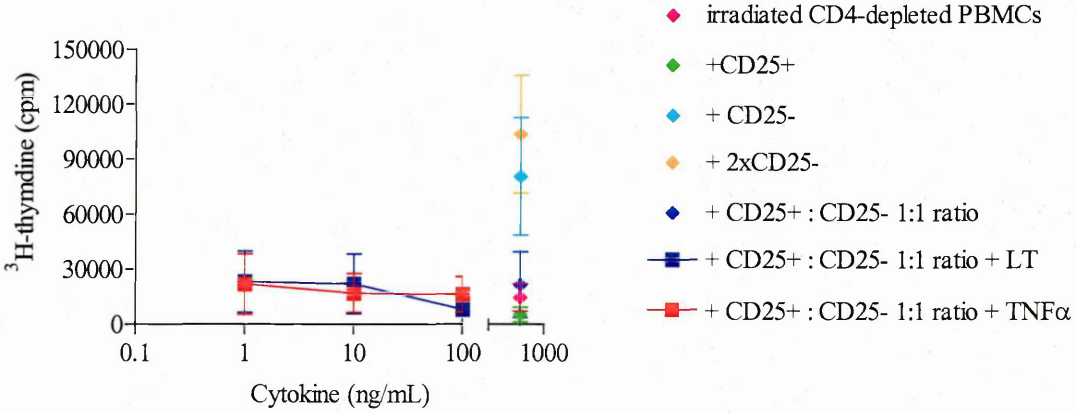


Figure 3.7b Investigating the ability of exogenous TNFα or LT to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. Exogenous human TNFα or LT was added at the start of the assay at the concentrations indicated. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors.

3.2.5 Establishment of mouse CD4+CD25+ suppressor assays

The addition of IL-2 had no significant effect on the ability of human CD4+CD25+ T cells to regulate CD4+CD25- T cells. It was decided to investigate whether the same effect was observed with mouse CD4+CD25+ regulatory cells. Much of the reported data investigating the effect of IL-2 on reversing suppression had been conducted using mouse CD4+CD25+ regulatory T cells (Shevach, 2002). Mouse CD4+CD25+ T cells were purified from mouse spleens using a two-step MACS magnetic bead system. Initially the CD4+ T cells were isolated by negative selection followed by a positive selection for CD25. Analysis by flow cytometry of the purified CD4+CD25+ and CD4+CD25- T-cell populations, is shown in figure 3.8. Following purification, a good enrichment of CD25 positive cells was obtained with 78% of the cells expressing high levels of CD25. The purity of the CD4+CD25- and CD4+CD25+ populations was 98% and 96% with respect to CD4 expression.

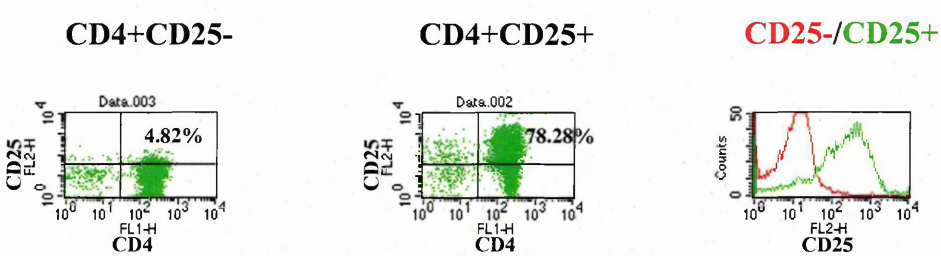


Figure 3.8 Isolation of mouse splenic CD4+CD25+ regulatory T cells.
CD4+ T cells were isolated from splenocytes using a MACS CD4+ negative selection isolation kit and separated into CD25+ and CD25- fractions using a MACS CD25 positive selection isolation kit. The purified CD4+ T cell populations were analysed by flow cytometry. Data is representative of one of 5 experiments.

Purified CD4+CD25+ T cells were co-cultured with purified CD4+CD25- cells in the presence of irradiated CD4-depleted splenocytes and anti-CD3. As can be seen in figure 3.9, the purified CD4+CD25+ cells significantly suppressed the proliferative response of the CD4+CD25- cells in response to anti-CD3 stimulation.

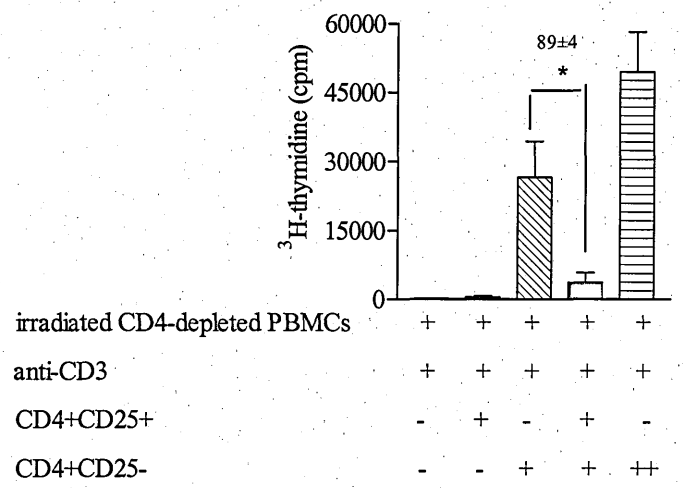


Figure 3.9 Inhibition of proliferation of mouse CD4+CD25- responder cells by mouse CD4+CD25+ regulatory cells following stimulation with anti-CD3.
Purified CD4+CD25- T cells were stimulated with anti-CD3 and irradiated CD4-depleted splenocytes in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 5-day culture period. Data shown are mean ± SEM of 5 mice. Value above bar indicates percentage inhibition ± SEM.*, $p<0.05$, paired t-test.

The addition of exogenous mouse IL-2 to the mouse suppression assay had a dramatic effect. As is illustrated in figure 3.10, the CD4+CD25+ mediated suppression of the CD4+CD25- T cells was completely reversed following the addition of 10ng/mL (20U/mL) exogenous IL-2. This effect is in sharp contrast to the data obtained with human CD4+CD25+ T cells and suggests a fundamental difference in the CD4+CD25+ regulatory T cells between the two species. One possible explanation for this difference is the source of cells used. The human CD4+CD25+ cells were isolated from peripheral blood whereas the mouse CD4+CD25+ cells were isolated

from spleen. However due to the availability of material, it was not possible to repeat the studies using mouse blood or human spleen and hence this could not be determined.

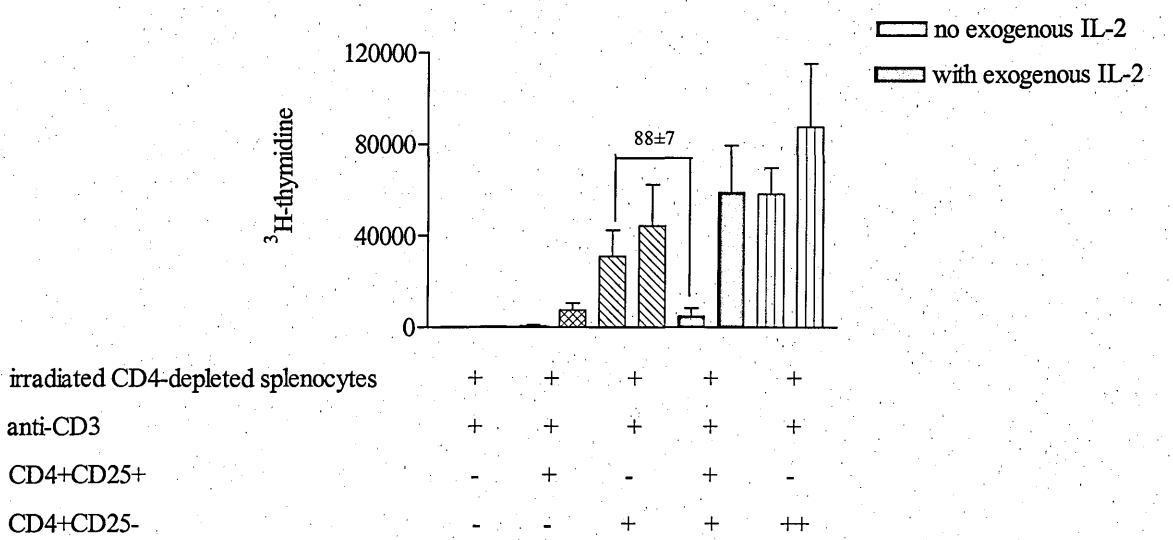


Figure 3.10 Reversal of CD4+CD25+ mediated suppression following the addition of exogenous mouse IL-2.
Purified CD4+CD25- T cells were stimulated with anti-CD3 and irradiated CD4-depleted splenocytes in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10ng/mL mouse IL-2 was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 5-day culture period. Data shown are mean ± SEM of 3 mice. Value above bar indicates percentage inhibition ± SEM

3.2.6 Investigation into whether IL-10 and TGFβ play a role in vitro

To further characterise the cells used in this study, neutralising antibodies to human IL-10 and human TGF-β1, -β2 , -β3 (TGFβ) were added to the assay. The results illustrated in figures 3.11a and b clearly show that neutralising IL-10 or TGFβ had no effect on the ability of the CD4+CD25+ cells to suppress the CD4+CD25- responder cells.

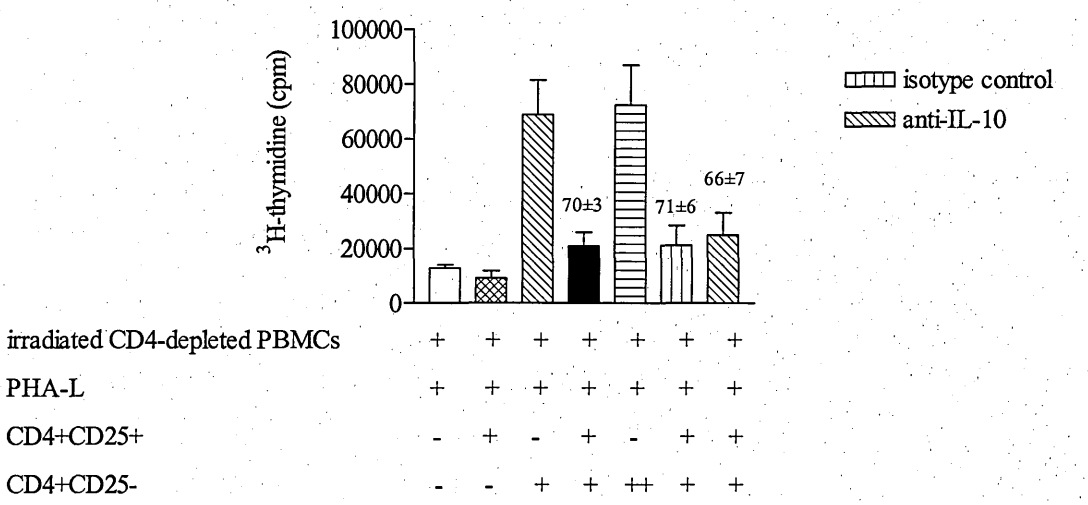


Figure 3.11a Investigating the ability of a neutralising anti-IL-10 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-IL-10 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

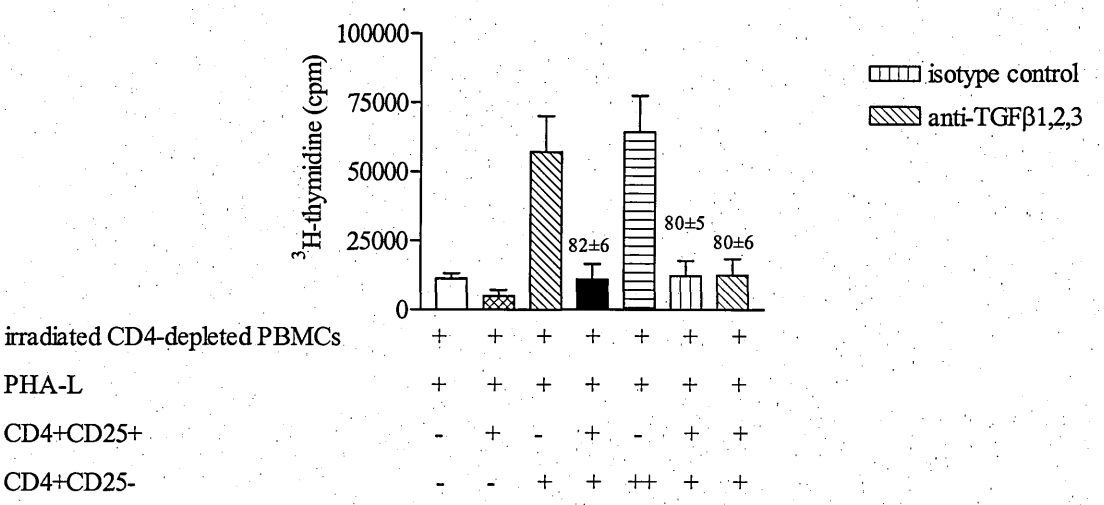


Figure 3.11b Investigating the ability of a neutralising anti-TGFβ1,2,3 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-TGFβ 1,2,3 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

3.2.7 Analysis of cell surface marker expression – resting cells versus activated cells

In an attempt to clarify which markers are most appropriate for human CD4+CD25+ T cells and also to identify markers that might play a role in regulatory T cell function, a full analysis of cell surface marker expression was carried out by flow cytometry of both the CD4+CD25+ regulatory T cell population and the CD4+CD25- responder T cells population. Both freshly isolated cells and cells activated for three days in the presence of PHA-L and irradiated CD4-depleted PBMCs were analysed.

Figure 3.12 illustrates some of the data obtained from the analysis of freshly isolated cells. Each dot-plot is representative of one of three donors analysed. The figures given above each dot-plot represent the mean percentage positive cells in the upper right quadrant ± SEM of 3 donors. In addition to CD25, both CD95 (Fas) and tumour necrosis factor receptor (TNFR) 2 were upregulated on the resting CD4+CD25+ T cell population compared to the CD4+CD25- T cell population. The percentage of CD4+CD25+ cells that expressed CD95 was 84.7 ± 1.6% compared to 38.9 ± 4.5% for the CD4+CD25- cells. Expression of TNFR2 on the surface of CD4+CD25+ T cells was 39.6 ± 9.8% compared to 9.3 ± 0.8% for the CD4+CD25- population. Levels of intracellular CTLA-4 were also higher for the CD4+CD25+ population with 50.0 ± 15.9% of the CD25+ cells expressing intracellular CTLA-4 compared to 9.2 ± 6.3% of the CD25- cells. However the levels of cell surface CTLA-4 were low for both populations, i.e. less than 1%.

Expression of CD45RO was higher on the CD4+CD25+ cells (92.8 ± 1.4% versus 62.9 ± 4.1%) and conversely the levels of CD45RA were lower (48.3 ± 7.7% versus 74.2 ± 7.6%). There were also a few CD4+CD25+ T cells that expressed high levels of inducible costimulator (ICOS) (< 10%) and CD103 (<5%). In contrast to reports in the literature, the data summarised in table 3.1 shows that the levels of GITR, CD134 (OX40), blood dendritic cell antigen (BDCA)-4, (neuropilin-1), galectin-1, programmed death (PD)-1 and CD137 (4-1BB) were not elevated on the freshly isolated CD4+CD25+ T cell population in comparison to the freshly isolated CD4+CD25- population.

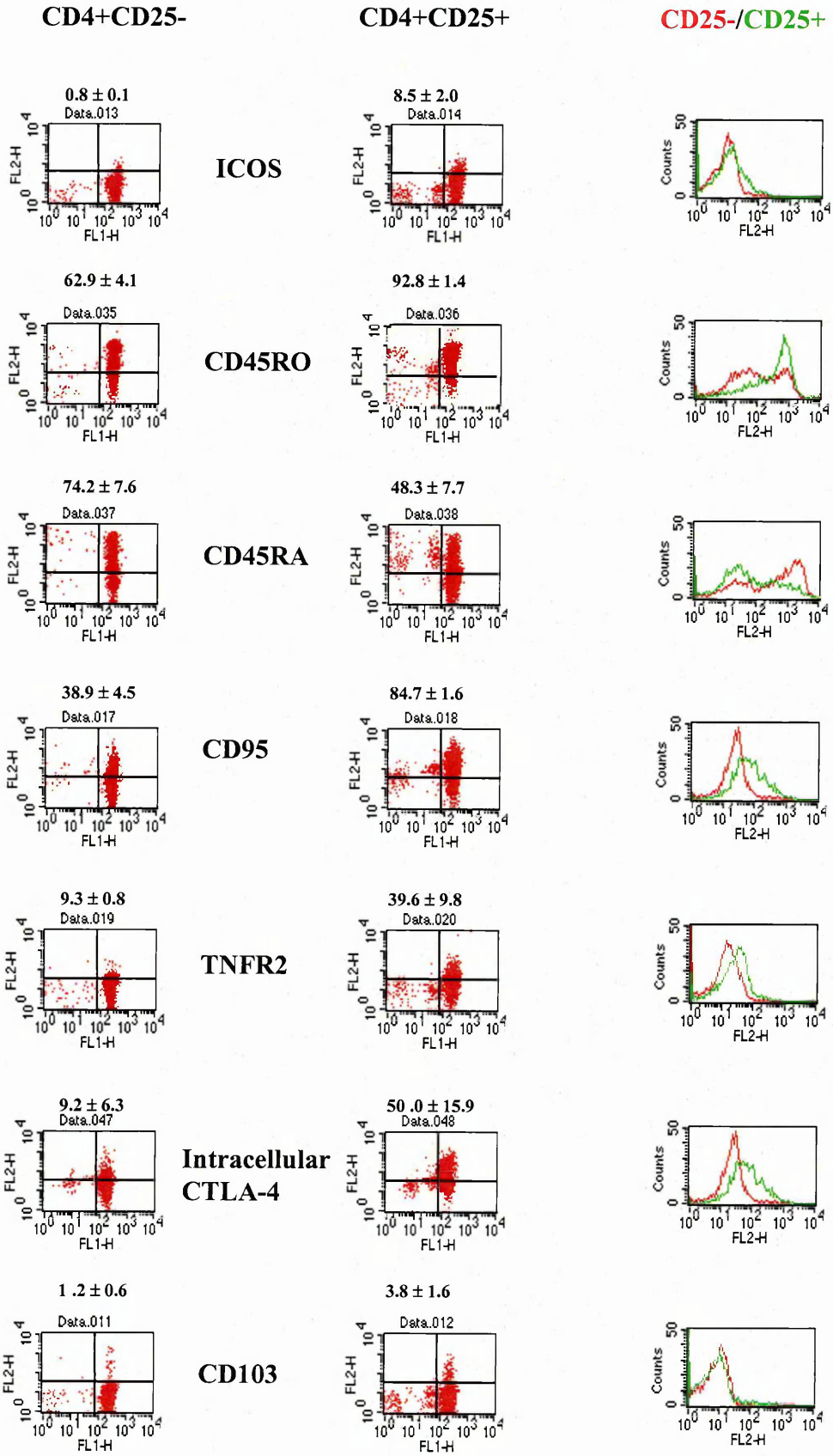


Figure 3.12 Characterisation of cell surface molecules expressed on freshly isolated CD4+CD25- and CD4+CD25+ T cells.

Cells were stained with CD4-FITC (FL1) and a PE-labelled antibody to the surface marker of interest (FL2) and then analysed by flow cytometry. Dot plots are representative of 1 of 3 donors analysed. Values represent the mean percentage positive cells in the upper right quadrant ± SEM of 3 donors.

Only a limited number of differences in cell surface marker expression were noted between the two cell populations following activation in the presence of irradiated CD4-depleted PBMCs and PHA-L. Figure 3.13 highlights three of the surface molecules that did appear to show differential expression. The activated CD4+CD25+ regulatory cells expressed higher levels of TNFR2 expression (86.1 ± 4.8% of the cells were positive compared to 46.4 ± 7.1% of the activated CD4+CD25- population). Conversely the number of activated CD4+CD25+ cells that expressed CD40L was lower and there were fewer CD4+CD25+ cells expressing CD45RA. Analysis of the remaining cell surface markers is summarised in table 3.1. Levels of intracellular CTLA-4 remained high in the CD4+CD25+ regulatory population even after activation (91.4 ± 1.4% versus 62.8 ± 4.7% for the activated CD4+CD25- population).

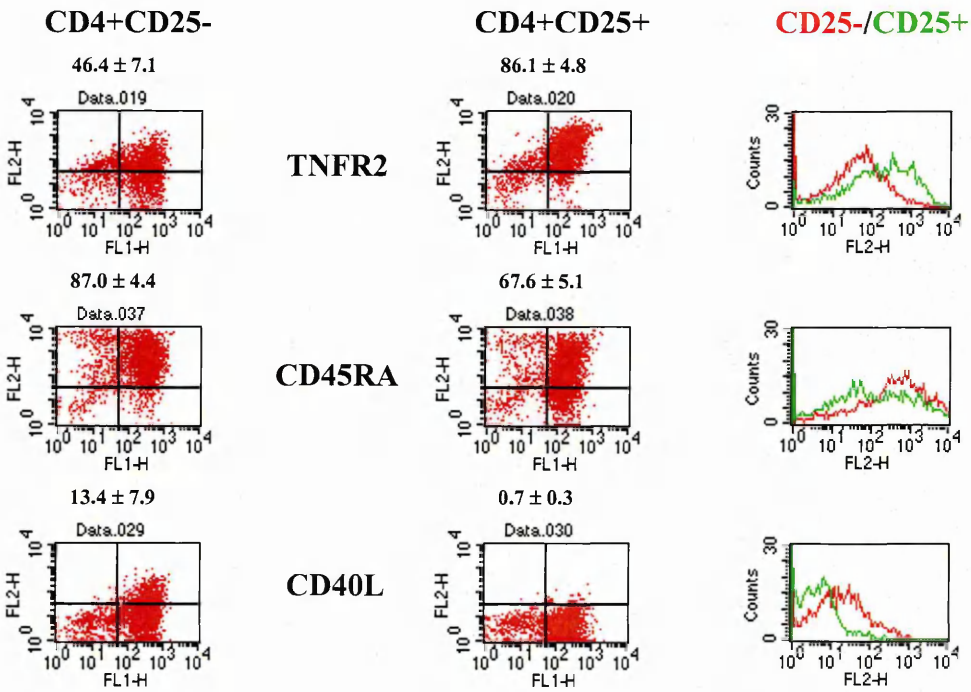


Figure 3.13 Characterisation of cell surface molecules expressed on the surface of activated CD4+CD25- and CD4+CD25+ T cells.
Cells were activated for 3 days in the presence of CD4-depleted PBMCs and PHA-L. Cells were then stained with CD4-FITC (FL1) and a PE-labelled antibody to the surface marker of interest and analysed by flow cytometry. Dot plots are representative of 1 of 3 donors analysed. Values represent the mean percentage positive cells in the upper right quadrant ± SEM of 3 donors.

Cell surface marker	Freshly isolated CD4+ T cells		3 day activated CD4+ T cells	
	CD25-	CD25+	CD25-	CD25+
BDCA-4	0.3 ± 0.2	2.0 ± 1.1	5.3 ± 1.2	7.0 ± 0.6
CD27	93.2 ± 2.2	95.3 ± 1.8	81.5 ± 67	89.2 ± 4.6
CD25	19.3 ± 3.7	87.1 ± 1.9	90.8 ± 2.9	92.6 ± 1.9
CD40L	0.1 ± 0.1	0.2 ± 0.1	13.4 ± 8.0	0.7 ± 0.3
CD45RA	74.2 ± 7.6	48.3 ± 7.7	87.0 ± 4.4	67.6 ± 5.1
CD45RO	62.9 ± 4.1	92.8 ± 1.4	95.5 ± 0.6	95.9 ± 1.3
CD62L	88.1 ± 5.8	93.3 ± 3.8	76.4 ± 4.6	88.8 ± 3.2
CD69	0.1 ± 5.5	0.6 ± 0.1	47.0 ± 1.9	45.5 ± 2.9
CD95	38.9 ± 4.5	84.7 ± 1.6	95.1 ± 1.5	94.9 ± 0.6
CD103	1.2 ± 0.6	3.8 ± 1.6	2.1 ± 0.8	6.3 ± 2.8
CD122	19.5 ± 2.5	32.3 ± 5.9	50.1 ± 16.5	68.8 ± 10.3
CD132	9.8 ± 5.6	6.7 ± 2.2	27.9 ± 14.6	16.9 ± 5.2
CD134	0.5 ± 0.3	1.8 ± 1.1	75.8 ± 10.7	64.3 ± 1.5
CD137	0.2 ± 0.2	1.3 ± 0.8	26.0 ± 9.4	25.7 ± 1.7
CD152 (CTLA-4, intracellular)	9.2 ± 6.3	50.0 ± 15.9	62.8 ± 4.7	91.4 ± 1.4
CD152 (CTLA-4, surface)	0.1 ± 0	0.1 ± 0	14.3 ± 6.4	14.0 ± 1.6
Galectin-1	0.1 ± 0	0.1± 0.1	0.4 ± 0.3	2.7 ± 1.5
GITR	0.3 ± 0.1	1.5 ± 1.2	51.7 ± 8.0	54.0 ± 2.0
ICOS	0.8 ± 0.1	8.5 ± 2.0	51.3 ± 15.7	33.7 ± 4.3
PD-1	5.1 ± 1.8	3.1 ± 0.7	39.0 ± 6.2	20.7 ± 2.3
TNFR2	9.3 ± 0.8	39.6 ± 9.8	46.4 ± 7.1	86.1 ± 4.8

Table 3.1 Summary of data comparing the expression levels of various cell surface markers on freshly isolated peripheral blood derived and 3 day activated CD4+CD25- and CD4+CD25+ T cells.

Cells were stained with CD4-FITC and a PE-labelled antibody to the surface marker of interest and analysed by flow cytometry. Data is expressed as the percentage positive CD4+ T cells for the cell surface marker of interest. Data shown are mean ± SEM of 3 donors.

3.2.8 Investigation into the role of IL-2 and its receptor

The regulatory T cells isolated in this study were purified on the basis of CD25 expression. Indeed CD25 is generally accepted as the most reliable marker of a resting regulatory T-cell. However whether CD25 itself plays a role in regulatory T-cell function is unclear. Data published from *in vitro* studies in the mouse showed that IL-2 released early following activation of CD4+CD25- cells, was required by the CD4+CD25+ T cells before they were able to regulate (Thornton *et al.*, 2004). These data suggest that the high levels of expression of CD25 might indeed be important for function. To address this question, experiments were conducted looking at the effect of a neutralising antibody to IL-2 and blocking antibodies to both CD25 (IL-2Rα) and CD122 (IL-2Rβ). The data shown in figures 3.14a-c demonstrate no significant effect of any of the antibodies on the ability of the CD4+CD25+ T cells to regulate.

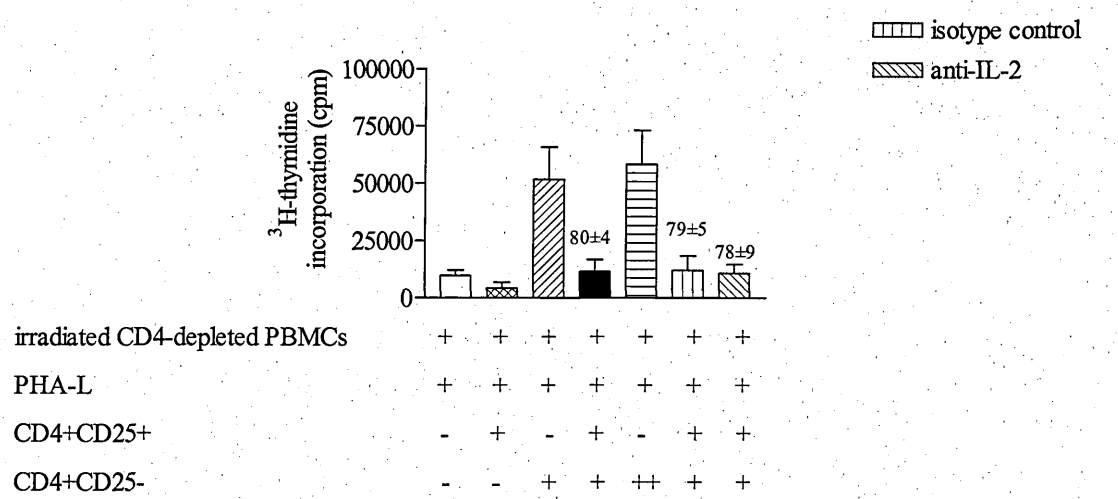


Figure 3.14a Investigating the ability of a neutralising anti-IL-2 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-IL-2 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

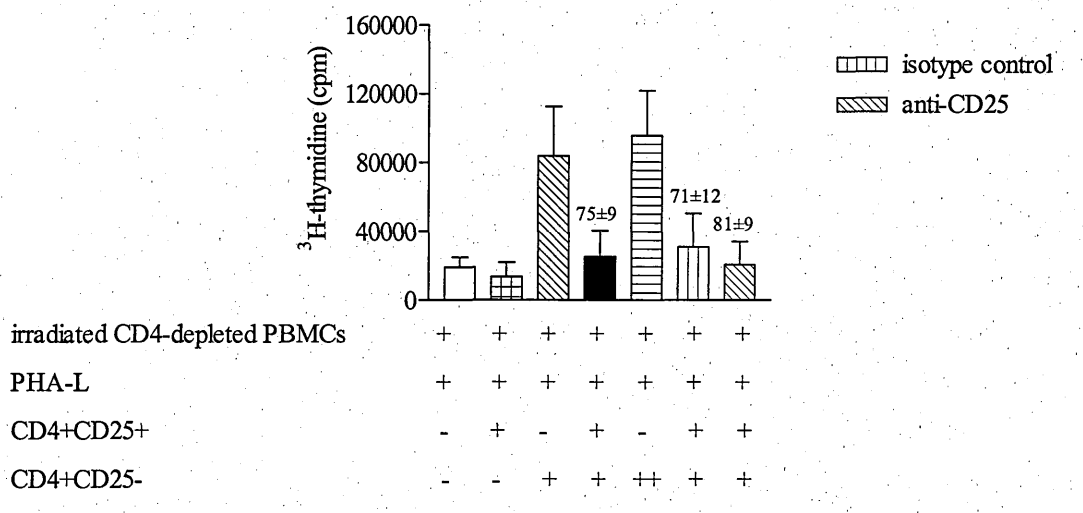


Figure 3.14b Investigating the ability of a blocking anti-CD25 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-CD25 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

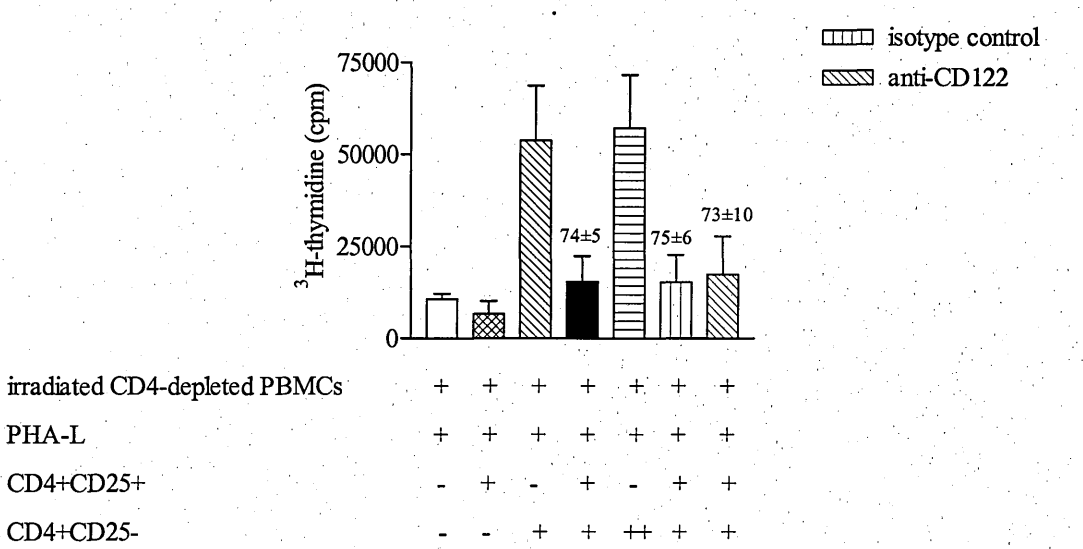


Figure 3.14c Investigating the ability of a blocking anti-CD122 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-CD122 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

3.2.9 Investigation into the role of CTLA-4

CTLA-4 is a key negative regulator of T-cell activation. The molecule is also upregulated, at least at the intracellular level, on CD4+CD25+ cells. However it is unclear whether this molecule plays a role in regulatory T-cell function. In an attempt to address this, a blocking antibody to human CTLA-4 was added to the suppression assay (figure 3.15a). Conversely, an agonising anti-CD28 antibody was also assessed (figure 3.15b). Perhaps somewhat surprisingly, neither of the antibodies had any significant effect on the CD4+CD25+ mediated suppression. This suggests that CTLA-4 does not play a significant role in contributing to the inhibition of CD4+CD25- T-cell proliferation in this assay system. However one reagent that did have a significant effect on the suppression assay was mouse CTLA-4-Ig fusion protein. Addition of this reagent to the suppressor assay caused a significant augmentation of the suppressor response (figure 3.15c). CD80 and CD86 are both ligands for CD28 and are responsible for delivering the second signal in T cell activation (Alegre *et al.*, 2001). However these molecules are also ligands for CTLA-4 and when engaged by CTLA-4, T-cell activation is inhibited. Therefore one possible explanation for the augmented inhibition of the response may be that the fusion protein preferentially binds CD80 and CD86, resulting in a reduced costimulatory signal being delivered through CD28 on the responder cell. This reduction in second signal leads to lower levels of activation of the CD4+CD25- cells resulting in reduced levels of proliferation by the CD4+CD25- T cell population.

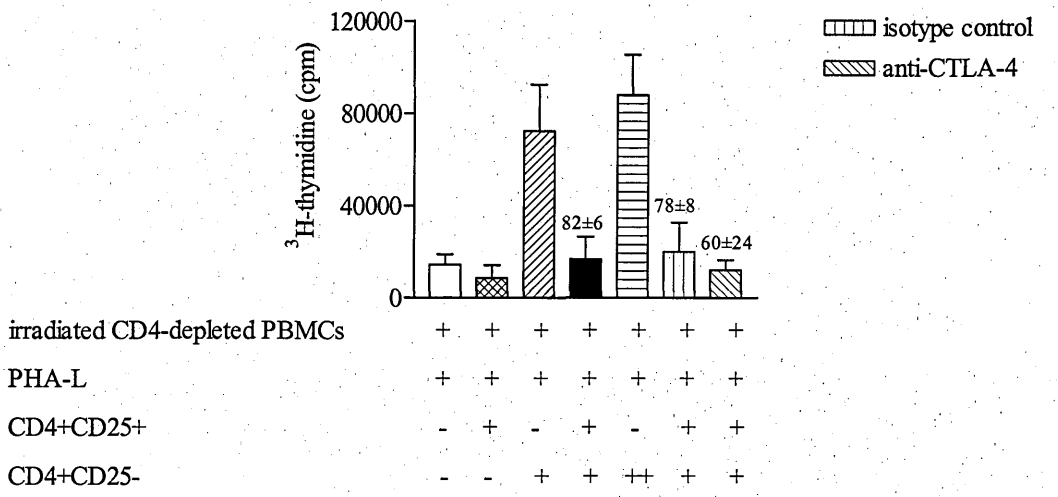


Figure 3.15a Investigating the ability of a blocking anti-CTLA-4 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-CTLA-4 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 5 donors. Values above bars indicate percentage inhibition ± SEM.

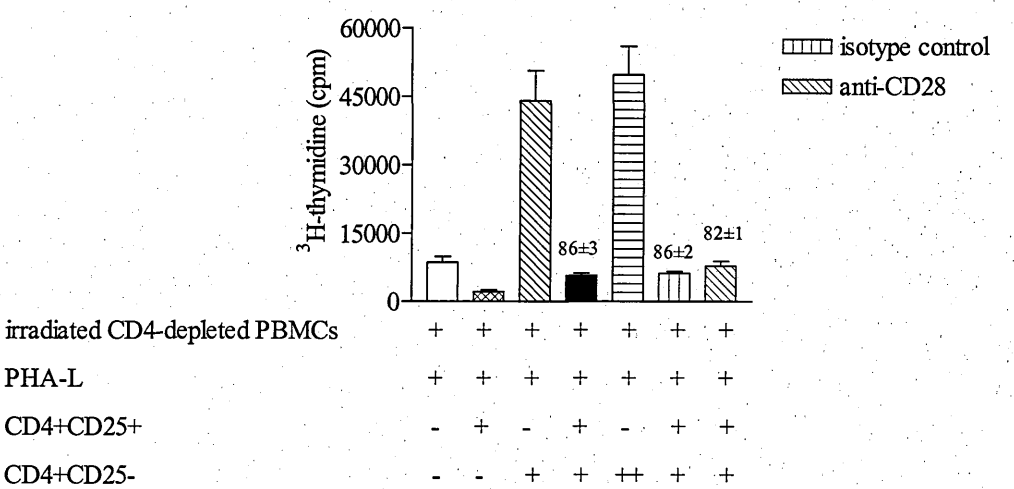


Figure 3.15b Investigating the ability of an activating anti-CD28 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-CD28 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

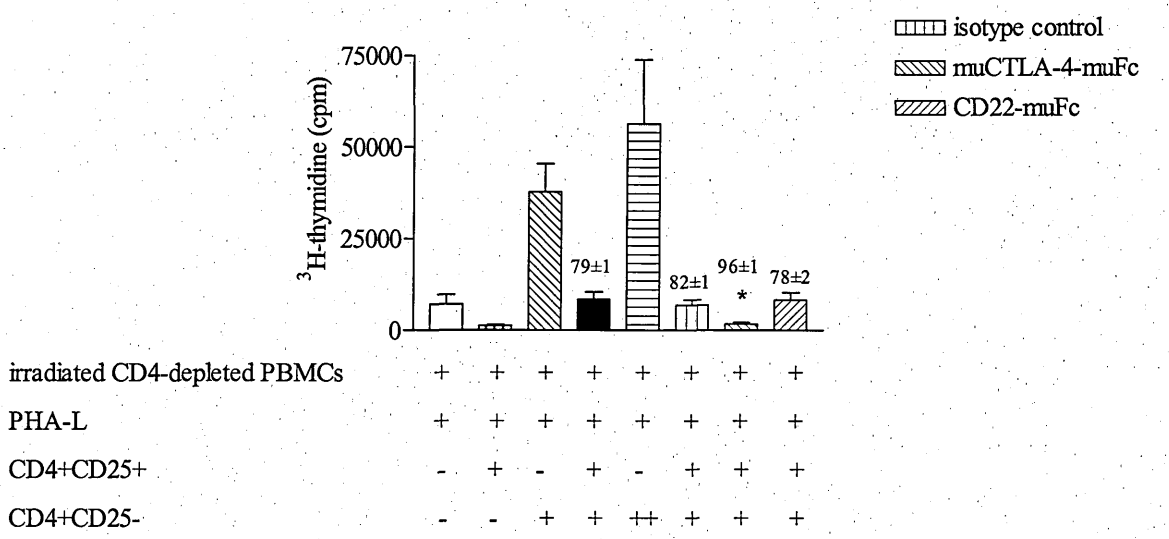


Figure 3.15c Investigating the effect of muCTLA-4-muFc on CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of muCTLA-4-muFc fusion protein or an isotype matched control antibody (MOPC21) or a control fusion protein (CD22-muFc) was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. *, $p<0.05$, paired t-test as compared to either MOPC21 or CD22-muFc. Values above bars indicate percentage inhibition ± SEM.

3.2.10 Attempts to identify cell surface molecules involved in CD4+CD25+ T cell mediated suppression

The data presented thus far have failed to identify a key cytokine or cell surface molecule that is solely responsible for mediating human CD4+CD25+ T-cell suppression. These data have concentrated on some of the most likely candidates based on current published data. However, there are other potential candidates so further experiments were conducted using antibodies to some of the other potential targets that could be involved in CD4+CD25+ mediated suppression. Unfortunately none of the antibodies investigated had any effect on the ability of the CD4+CD25+

cells to suppress the proliferative response of the CD4+CD25- cells following stimulation with PHA-L. The results obtained are summarised in table 3.2.

Antibody	Isotype	Mechanism of action	Reversal of suppression
Anti-CD134	Mouse IgG1	Blocks ligand binding	No effect
Anti-GITR	Mouse IgG1	Blocks ligand binding	No effect
Anti-LAG-3	Mouse IgG1	Blocks ligand binding	No effect
Anti-TGFβRII	Goat IgG	Blocks TGFβ binding	No effect
Anti-IL-17	Mouse IgG2b	Neutralises bioactive IL-17	No effect

Table 3.2 Summary of data investigating the ability of different antibodies to reverse CD4+CD25+ mediated suppression of CD4+CD25- cells following activation with PHA-L.

3.2.11 Purification of regulatory T cells on the basis of either CD95 expression or TNFR2 expression

Characterisation of freshly isolated CD4+CD25+ cells by flow cytometry showed elevated levels of both CD95 and TNFR2 as compared to freshly isolated CD4+CD25- T cells (figure 3.12). One explanation for these elevated levels could be that one or both of these molecules play a role in regulatory T-cell function. It was decided initially to purify cells from the CD4+ T-cell population on the basis of either CD95 or TNFR2 expression and to investigate whether these cells had the ability to suppress. Human CD4+CD95+ T cells were purified from PBMCs using a two-step MACS magnetic bead system. The CD4+ T cells were isolated by negative selection followed by a positive selection for CD95 or TNFR2 using either an anti-CD95 PE-labelled antibody, or an anti-TNFR2 PE-labelled antibody and anti-PE microbeads.

Following purification, the CD4+ T cell populations were stained for CD25 expression levels. Analysis by flow cytometry of the purified CD4+ populations is shown in figure 3.16.

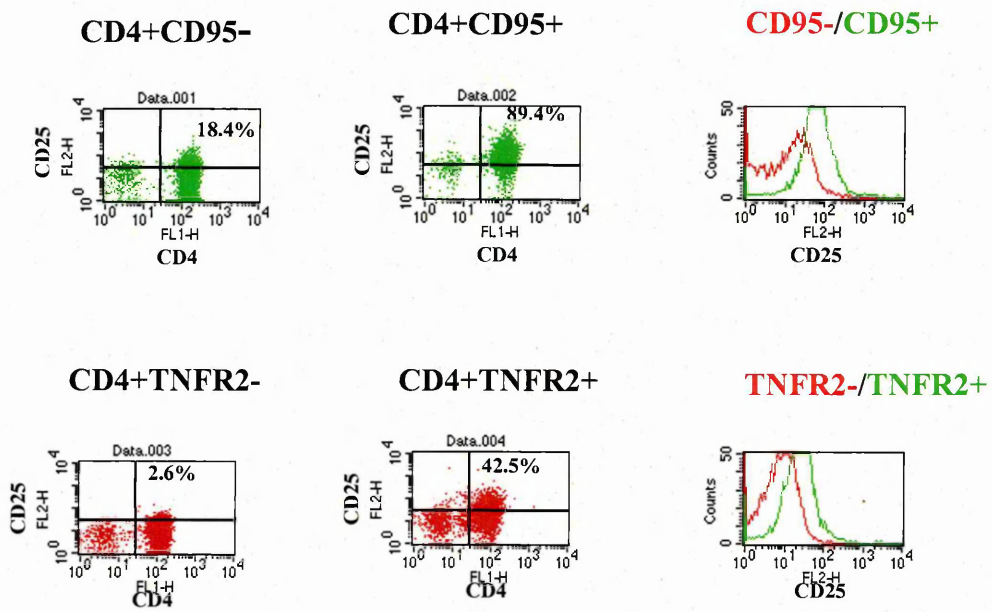
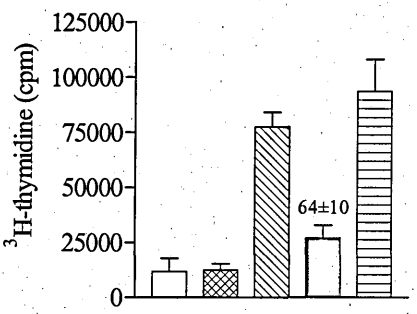


Figure 3.16 Isolation of human CD4+ T cells by positive selection for CD95 or TNFR2 expression for assessment of regulatory function.
CD4+ T cells were isolated from PBMCs using a MACS CD4+ negative selection isolation kit and separated into CD95+, CD95- or TNFR2+ and TNFR2- fractions using the appropriately labelled PE antibody and anti-PE microbeads. The purified CD4+ T cells were analysed for CD25 expression by flow cytometry. Percentage values for the number of CD25+ cells are displayed. Data shown are of one of two donors analysed.

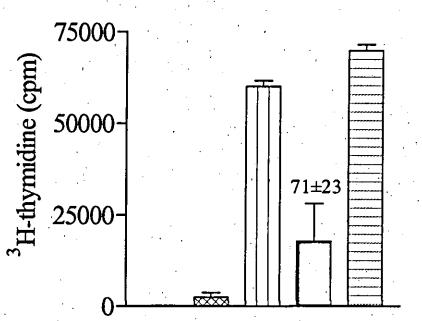
The dot plots in figure 3.16 show that the CD4+CD95+ T cells expressed higher levels of CD25 than the CD4+TNFR2+ cells, 89 % versus 43%. Functional assays were set up to investigate the regulatory capacity of these populations. Purified CD4+CD95+ T cells were co-cultured with purified CD4+CD95- cells in the presence of irradiated CD4-depleted PBMCs and either PHA-L or tetanus toxoid. Identical assays were set up with CD4+TNFR2+ and CD4+TNFR2- T cells using cells isolated from the same donor. The results obtained with the CD95 selected cells are illustrated in figures 3.17a and b and the TNFR2 selected cells in figures 3.18a and b. Interestingly the results from the functional assays showed that the CD4+TNFR2+ T

cells achieved significant levels of suppression compared to the CD4+CD95+ T cells, even though the CD4+TNFR2+ cells expressed lower levels of CD25. Unfortunately due to limits on the amount of blood that can be taken from any one donor, it was not possible to isolate cells on the basis of CD25 expression and compare the levels of suppression achieved in the same experiment with the CD95+ and TNFR2+ enriched cells. However the TNFR2 data appeared comparable to data obtained with CD25+ isolated cells (figure 3.2a) with approximately 80% inhibition being achieved by both cell populations.



irradiated CD4-depleted PBMCs	+	+	+	+	+
PHA-L	+	+	+	+	+
CD4+CD95+	-	+	-	+	-
CD4+CD95-	-	-	+	+	++

Figure 3.17a Inhibition of proliferation of CD4+CD95- responder cells by CD4+CD95+ regulatory cells following stimulation with PHA-L.
Purified CD4+CD95- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD95+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.



irradiated CD4-depleted PBMCs	+	+	+	+	+
Tetanus toxoid	+	+	+	+	+
CD4+CD95+	-	+	-	+	-
CD4+CD95-	-	-	+	+	++

Figure 3.17b Inhibition of proliferation of CD4+CD95- responder cells by CD4+CD95+ regulatory cells following stimulation with tetanus toxoid.
Purified CD4+CD95- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+CD95+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 7-day culture period. Data shown are mean of 2 donors. Error bars represent minimum and maximum values. Values above bars indicate percentage inhibition ± SD.

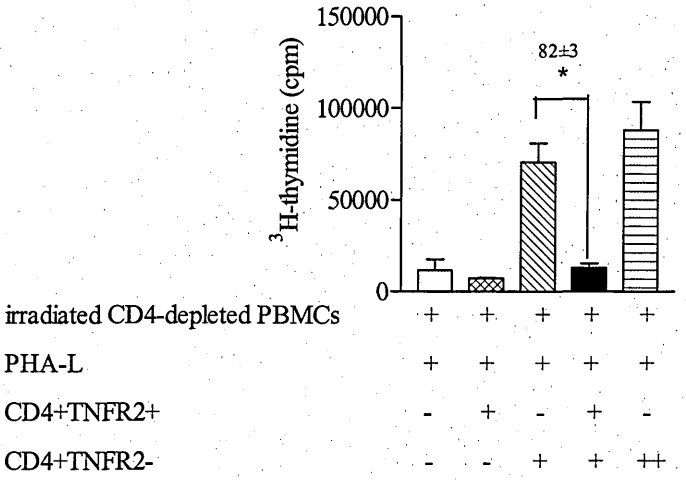


Figure 3.18a Inhibition of proliferation of CD4+TNFR2- responder cells by CD4+TNFR2+ regulatory cells following stimulation with PHA-L.
Purified CD4+TNFR2- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+TNFR2+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM. *, $p<0.05$, paired t-test.

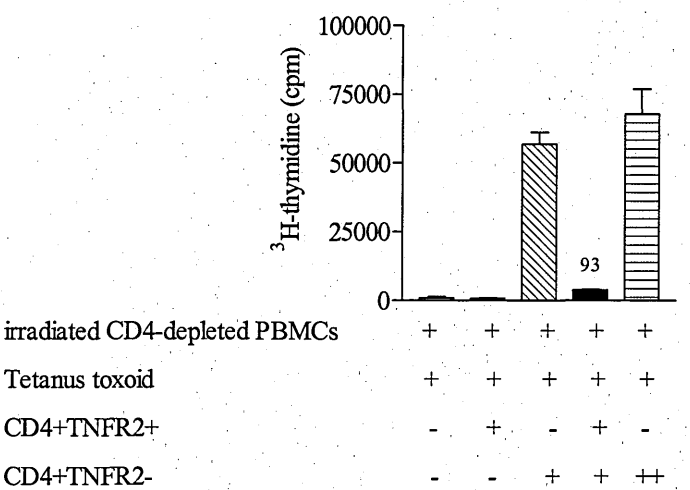


Figure 3.18b Inhibition of proliferation of CD4+TNFR2- responder cells by CD4+TNFR2+ regulatory cells following stimulation with tetanus toxoid.
Purified CD4+TNFR2- T cells were stimulated with tetanus toxoid and irradiated CD4-depleted PBMCs in the presence of CD4+TNFR2 T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 7-day culture period. Data shown are mean of 2 donors. Error bars represent minimum and maximum values. Values above bars indicate percentage inhibition.

The functional assays described have revealed that CD4+ regulatory cells isolated on the basis of CD95 or TNFR2 expression are capable of suppressing responder CD4+ T cell proliferation. However the main question to be addressed was whether either of these molecules played a role in regulatory T cell function. To investigate this, antibodies to TNFR2 and CD95 were added to the suppressor T cell assays. The CD4+ regulatory cells used in these assays were purified on the basis of CD25.

The data illustrated in figures 3.19 and 3.20 clearly shows that neither the blocking antibodies to CD95 and TNFR2, nor the apoptosis-inducing anti-CD95 antibody had any effect on the ability of the CD4+CD25+ cells to inhibit the proliferation of the CD4+CD25- cells. These data suggest that *in vitro* at least, the mechanism by which CD25+CD4+ T cells suppress CD4+CD25- T cells is not dependent on TNFR2 or CD95. One might have expected the apoptosis-inducing anti-CD95 antibody to preferentially deplete the CD4+CD25+ cells and hence show signs of reversal of suppression. However this antibody requires cross-linking for apoptosis to be induced (Huang *et al.*, 1999) and it is possible that optimal cross-linking did not occur in this assay, or the cells may still be able to suppress even if they are undergoing apoptosis.

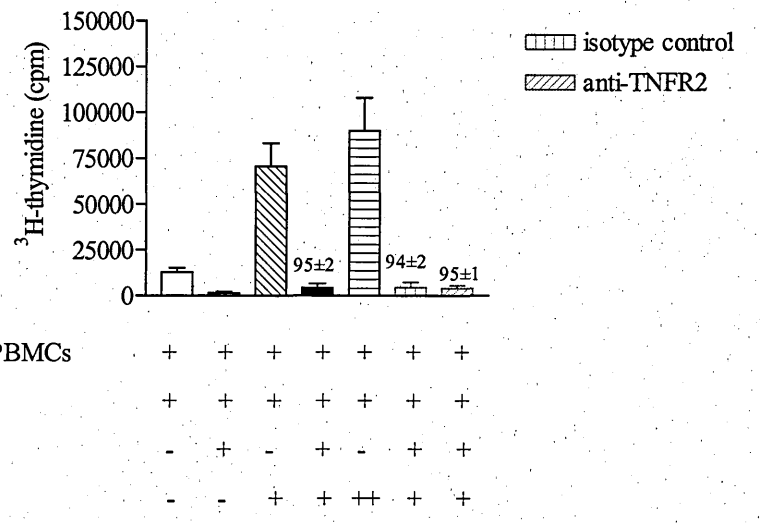


Figure 3.19 Investigating the ability of a blocking anti-TNFR2 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L.
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-TNFR2 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

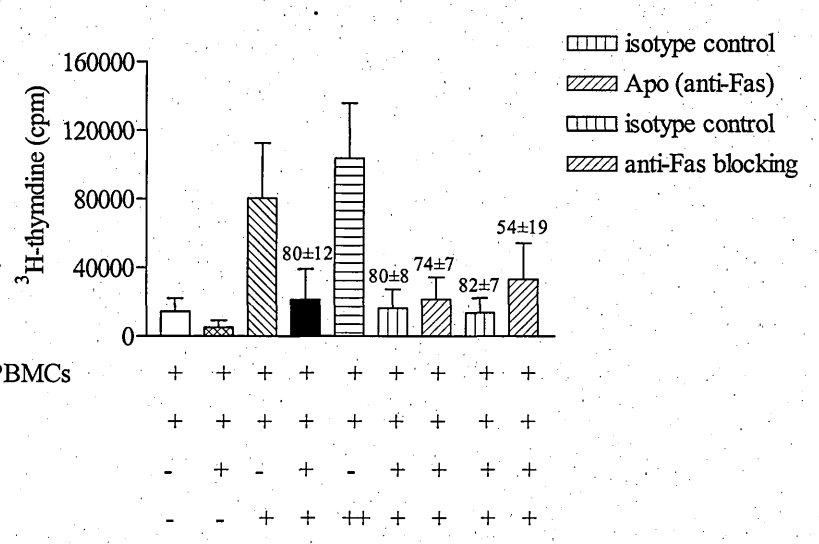


Figure 3.20 Investigating the ability of a blocking anti-CD95 antibody and an apoptosis-inducing anti-CD95 antibody to reverse CD4+CD25+ T cell mediated suppression following activation with PHA-L
Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD25+ T cells at a 1:1 ratio of T cells. 10μg/mL of an anti-CD95 or an isotype matched control antibody was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 3 donors. Values above bars indicate percentage inhibition ± SEM.

3.2.12 Investigation into the effect of positive selection

The experimental data presented thus far has shown that human CD4+ T cells positively selected on the basis of CD25, TNFR2 or CD95 cell surface expression, have the ability to suppress the proliferation of CD4+ T cells expressing low levels of the reciprocal marker. This is believed to be due to the isolation of a specific sub-population of regulatory CD4+ T cells, characterised by the cell surface expression of these markers, which have the ability to inhibit activation of CD4+ responder T cells. An alternative explanation is that it is the method of isolation of these cells that somehow renders them suppressive. Whilst thought to be highly unlikely, it was an important issue to investigate. CD4+ T cells were purified by negative selection as previously described. The CD4+ T cells were then purified further into CD45RO+ and CD45RO- fractions using a CD45RO-PE labelled antibody and anti-PE microbeads. Functional assays were set up to investigate the regulatory capacity of these populations. Purified CD4+CD45RO+ T cells were co-cultured with purified CD4+CD45RO- cells in the presence of irradiated CD4-depleted PBMCs and PHA-L. The data illustrated in figure 3.21 shows that CD4+CD45RO+ T cells isolated using positive selection, do not have regulatory activity, thus confirming that positive selection alone is not sufficient to confer regulatory activity.

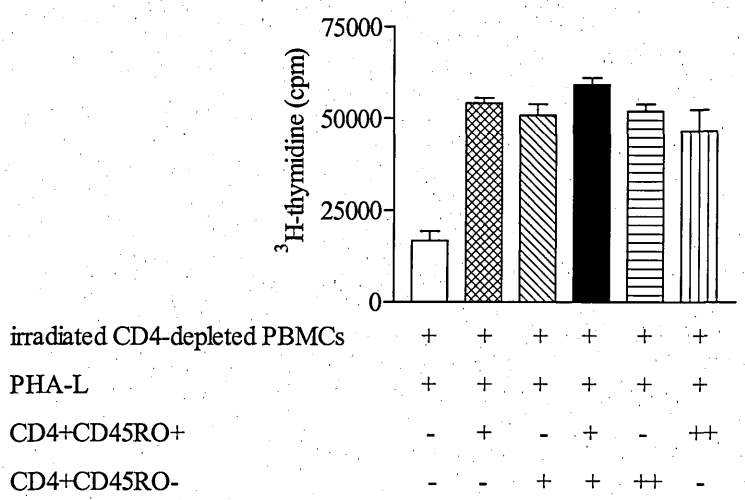


Figure 3.21 Investigating the ability of CD4+CD45RO+ T cells to inhibit CD4+CD45RO- T cell proliferation following activation with PHA-L.
Purified CD4+CD45RO- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs in the presence of CD4+CD45RO+ T cells at a 1:1 ratio of T cells. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown is representative of one of two donors analysed. Error bars represent SD of triplicate wells.

3.2.13 Investigation into the effect of antibodies to specific T cell surface markers on T cell proliferation.

One caveat with the studies described in this thesis, was the functionality of the antibodies used in the assays. Whilst the bioactivity of the cytokines has been confirmed in other cell based assays, a number of the antibodies used in these studies have not been evaluated in other cell-based assays and their reported function is solely based on the manufacturer's description. Therefore caution should be taken when analysing the results as a different antibody to the same target may have a different effect.

It was also possible that the antibodies were having an effect on the responder CD4+CD25- T cell population, which was masked in the coculture with the CD4+CD25+ regulatory T cells. In an attempt to address this, all the antibodies were screened to investigate their effect on the CD25- population alone, following stimulation with PHA-L in the presence of irradiated CD4-depleted PBMCs. The data shown in figure 3.22 summarises the results of one of two donors analysed. Interestingly none of the antibodies screened appeared to have a significant effect on responder CD4+ T cells proliferation. One might have expected the anti-IL-2 or anti-CD25 antibodies to affect proliferation however there are reports that strong stimulation through the TCR or with mitogens, only results in a partial inhibition of T cell activation using antibodies to IL-2 or CD25 (Malek and Bayer, 2004).

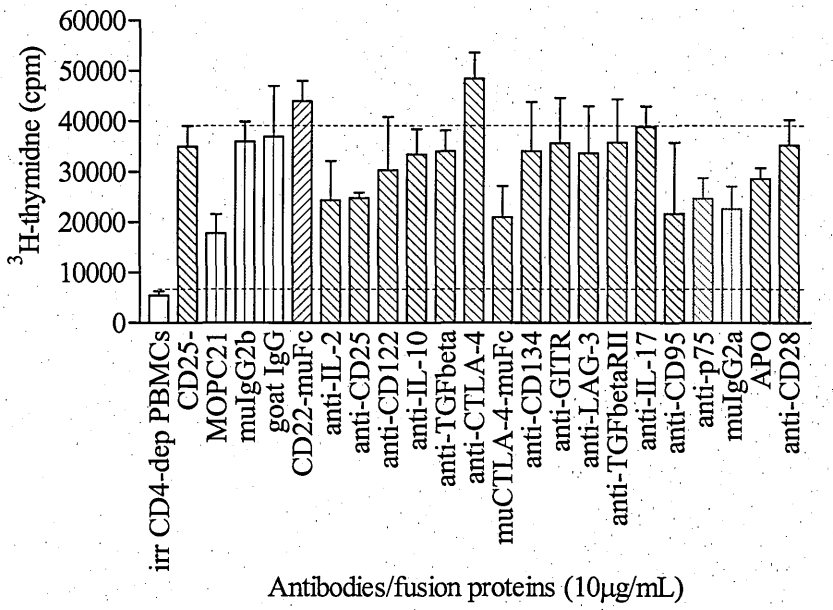


Figure 3.22 Investigating the effect of the antibodies and fusion proteins screened in the suppression assays on the CD4+CD25- T cell population alone, following stimulation with PHA-L.

Purified CD4+CD25- T cells were stimulated with PHA-L and irradiated CD4-depleted PBMCs. 10µg/mL of each antibody/fusion protein or isotype matched control antibody/fusion protein was added at the start of the assay. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown is representative of one of two donors analysed. Error bars represent SD of triplicate wells.

3.3 Discussion

The main aims of this chapter were to characterise the cell surface phenotype of peripheral blood derived human CD4+CD25+ regulatory T cells and to investigate the mechanism of action of these cells *in vitro*.

As already mentioned, it is widely accepted that naturally occurring CD4+ regulatory T cells express elevated levels of CD25. Further analysis of the surface phenotype confirmed that the CD4+CD25+ T cells isolated in these studies expressed high levels of CD95, TNFR2, CD45RO and intracellular CTLA-4. Subsequent isolation of CD4+ regulatory cells on the basis of either CD95 or TNFR2 expression, confirmed the ability of these cells to suppress T cell proliferation following stimulation with either PHA-L or tetanus toxoid. These data validate both CD95 and TNFR2 as robust cell surface markers for resting CD4+ regulatory cells. However, subsequent experiments also showed that whilst upregulated on the surface of CD4+CD25+ regulatory cells, these molecules did not appear to play a functional role *in vitro*.

Studies with human thymocytes have confirmed elevated levels of TNFR2 expression on CD4+CD25+ T cells (Annunziato *et al.*, 2002) and data from various groups have also shown upregulation of CD45RO and intracellular CTLA-4 (Taams *et al.*, 2001; Annunziato *et al.*, 2002; Dieckmann *et al.*, 2001; Ng *et al.*, 2001; Levings *et al.*, 2001; Jonuleit *et al.*, 2001). Studies showing upregulation of CD95 have led to the suggestion that CD4+CD25+ regulatory cells are a highly apoptosis prone and highly differentiated population (Taams *et al.*, 2001) This is supported by other data showing that these cells have shortened telomeres and low levels of CD45RB expression

(Taams *et al.*, 2002). Overall, these data are consistent with the idea that in humans at least, the CD4+CD25+ regulatory T-cell subset might not represent a unique lineage of cells derived from the thymus but instead they are a suppressive population of T cells derived from effector CD4+ T cells that have become highly differentiated (Akbar *et al.*, 2003).

Studies using human CD4+CD25+ thymocytes have confirmed however that they do exhibit regulatory activity (Annunziato *et al.*, 2002; Stephens *et al.*, 2001). Therefore it is conceivable that thymus-derived CD4+CD25+ T cells represent one type of regulatory cell and a second type of CD4+CD25+ T-cell may be derived in the periphery from fully differentiated effector T cells. Thus isolation of these cells from peripheral blood may contain a mixture of both regulatory cell types. Evidence for the generation of such CD4+CD25+ cells in the periphery is supported by data that has described the generation of FOXP3+ cells following activation of CD4+CD25- T cells with anti-CD3 and anti-CD28 mabs (Walker *et al.*, 2003a). Recent data from mouse *in vivo* studies has shown that peripheral mouse CD4+CD25- T cells were able to convert into CD4+CD25+ regulatory cells following transfer into normal recipients (Liang *et al.*, 2005).

In contrast to reports in the literature, the CD4+CD25+ regulatory cells isolated in these current studies did not appear to express elevated levels of CD103 (Lehmann *et al.*, 2002), OX40, galectin-1, GITR (Shimizu *et al.*, 2002; McHugh *et al.*, 2002), PD-1 or neuropilin-1 (Bruder *et al.*, 2004) when compared to CD4+CD25- cells. The number of CD4+CD25+ T cells expressing CD134 and GITR increased following activation, however this was also observed in the CD4+CD25- T cell population. The

cell surface molecules described above are primarily associated with mouse CD4+CD25+ regulatory cells, thus it is possible that these discrepancies are a result of species-specific or tissue-specific differences. However it is clear that currently none of the markers identified as being unique to CD4+CD25+ regulatory cells are truly reliable, as many of the molecules, including CD25, are upregulated on the surface of CD4+CD25- T cells following activation. Indeed it is highly likely that there is no single unique surface marker for these cells and maybe a combination of various cell surface and intracellular proteins determine the regulatory cell phenotype.

Following the observation that CD134 and GITR were not expressed at a higher level on CD4+CD25+ cells compared to CD4+CD25- T cells, it was not surprising to discover that neither of these molecules appeared to be responsible for suppressor function. This observation is supported by studies with anti-GITR antibodies that have shown reversal of suppression is only mediated with a whole antibody and not a Fab' fragment. Therefore suggesting that engagement of GITR with its ligand is not responsible for CD4+CD25+ mediated suppression (Shimizu *et al.*, 2002).

The titration experiment highlighted that maximal suppression *in vitro* was achieved when the ratio of responder cells to suppressor cells was 1:1 and as the numbers of suppressors declined, the level of inhibition also declined. This raises a question about how these cells can be effective at suppressing an immune response *in vivo* if their frequency is so low. More recent data generated from mouse studies, has shown that far from being refractory to TCR induced proliferation, these cells do undergo proliferation *in vivo* (Walker *et al.*, 2003b; Yamazaki *et al.*, 2003; Fisson *et al.*, 2003). There is also evidence that these cells may amplify their suppressive action *in vivo* by

acting on APCs to inhibit their maturation. Indeed coculture of activated CD4+CD25+ cells with APCs resulted in reduced expression of CD80 and CD86 (Cederbom *et al.*, 2000). Additionally, studies conducted *in vitro* have shown that coculture of immature APCs with CD4+ T cells can lead to the generation of IL-10 producing regulatory T cells (Jonuleit *et al.*, 2000).

Alternatively or concomitantly, the regulatory cells may interact with the APC *via* CTLA-4 and induce the upregulation of indoleamine 2,3-dioxygenase (IDO) resulting in tryptophan catabolism. This would lead to immune suppression resulting from a reduction in T-cell function and viability due to tryptophan depletion and the production of pro-apoptotic metabolites (Fallarino *et al.*, 2003; Grohmann *et al.*, 2003). Another mechanism for amplification of the suppressor response is provided by evidence that CD4+CD25+ cells have the ability to induce conventional T cells to become regulatory T cells via a mechanism known as infectious tolerance. These induced regulatory cells have been shown to suppress *via* the release of the immunomodulatory cytokines IL-10 or TGFβ (Stassen *et al.*, 2004). Together these mechanisms may help to explain some of the discrepancies observed regarding CD4+CD25+ functionality *in vitro* versus *in vivo*.

The experimental data presented in this chapter has highlighted how little is still known about the cell-cell contact mechanism of suppression of human CD4+CD25+ regulatory T cells *in vitro*. The addition of various cytokines and antibodies to cell surface molecules implicated in CD4+CD25+ T-cell function had no significant effect on reversing suppression. This is in sharp contrast to the data obtained using mouse

CD4+CD25+ regulatory T cells where suppression was fully reversible following the addition of exogenous IL-2.

Data from human *in vitro* systems regarding the ability of IL-2 to reverse suppression is mixed. Stephens *et al.* showed that human CD4+CD25+ thymocytes were responsive to IL-2 when it was added at 100U/mL (Stephens *et al.*, 2001). Conversely Annunziato *et al.* showed that human CD4+CD25+ thymocytes were not responsive to IL-2 (10U/mL) but suppression was reversed with exogenous IL-15 (10ng/mL) (Annunziato *et al.*, 2002). One could explain this discrepancy by arguing that Annunziato *et al.* may have seen reversal at higher concentrations of IL-2 but that does not account for the effect of IL-15.

Studies with human peripheral blood derived CD4+CD25+ cells are also unclear regarding the role of IL-2. Ng *et al.* showed full reversal of suppression with only 10U/mL of IL-2, however the overall suppression levels were only 50% (Ng *et al.*, 2001). Dieckmann *et al.* found partial reversal at only very high doses of IL-2 (100-1000U/mL). This response was improved if used synergistically with IL-15 (Dieckmann *et al.*, 2002). In agreement with that data, both Taams *et al.* (2001) and Jonuleit *et al.* (2001) showed partial reversal of suppression with IL-2, however Jonuleit *et al.* were able to show full reversal with a combination of IL-2 and IL-4. This suggested that the responder cell population was a mix of both Th1and Th2 cells and as such was responsive to different cytokines. Indeed data generated using Th1 and Th2 cell clones has shown that suppression of the Th2 clones was reversed following the addition of IL-4 but not IL-2. Interestingly, suppression of the Th1

clones could only be overcome by the addition of exogenous IL-15, not IL-2 (Cosmi *et al.*, 2004).

Data from the studies described earlier in this chapter have shown that the addition of exogenous IL-2 did not reverse PHA-L mediated suppression. The addition of exogenous IL-2 to the tetanus toxoid suppression assay resulted in a slight but not significant reversal of suppression. Reasons for this apparent unresponsiveness to IL-2 are unclear, however it would be interesting to evaluate cytokine combinations like IL-2, IL-15 and IL-4 in the same assay. The observation that suppression was reversed with exogenous IL-2 in the mouse assays is an interesting one. It is tempting to speculate that there might be intrinsic differences between human and mouse CD4+CD25+ T cells. This is also supported by the observed differences in cell surface marker expression between the human cells analysed in this study and the published data on mouse cell surface markers.

Experiments were conducted to investigate whether IL-10 and TGFβ were responsible for the CD4+CD25+ T cell mediated suppression. Blocking antibodies to both IL-10 and TGFβ and a blocking antibody to the TGFβRII receptor were added to the suppression assay, however no significant reversal of suppression was observed. This observation is in agreement with data generated from many groups and suggests that *in vitro*, soluble factors are not playing a role in CD4+CD25+ T cell mediated suppression (Jonuleit *et al.*, 2001; Taams *et al.*, 2001; Levings *et al.*, 2001; Thornton and Shevach, 1998). Evidence for the role of IL-10 and TGFβ in CD4+CD25+ T cell mediated suppression originates from *in vivo* studies. Studies in the SCID colitis model have shown that by blocking IL-10 signalling with an anti-IL-10 receptor

antibody, the suppressive function of $CD45RB^{low}$ cells is abrogated (Asseman *et al.*, 1999). Similarly $CD45RB^{low}$ cells isolated from IL-$10^{-/-}$ mice, failed to protect against colitis when cotransferred with $CD45RB^{hi}$ cells (Asseman *et al.*, 1999). However, CD4+CD25+ cells from IL-$10^{-/-}$ mice were fully competent suppressors *in vitro* (Thornton and Shevach, 1998).

Studies again in the SCID colitis model have shown that regulatory T-cell function can be blocked with neutralising anti-TGFβ antibodies (Powrie *et al.*, 1996; Fuss *et al.*, 2002). However again, CD4+CD25+ cells from $TGF\beta^{-/-}$ mice were functional suppressors *in vitro* (Piccirillo *et al.*, 2002). Why this discrepancy between *in vitro* and *in vivo* studies exists is unclear. One explanation could be due to differences in the complexities of the cellular environment and the different types of cells interacting with one another. Clearly the well of a tissue culture plate cannot substitute for a complex *in vivo* environment. Indeed, there is evidence that the release of IL-10 and TGFβ by CD4+CD25+ cells *in vivo* is responsible for suppressing the innate immune response (Maloy *et al.*, 2003). However interesting data has emerged suggesting a link for IL-10 and TGFβ *in vitro* through infectious tolerance. As mentioned earlier, studies by two groups have shown that CD4+CD25+ cells were able to induce a population of CD4+ T cells to produce either TGFβ (Jonuleit *et al.*, 2002) or IL-10 (Dieckmann *et al.*, 2002) following activation.

The CD4+CD25+ cells isolated in this study expressed high levels of intracellular CTLA-4, however a blocking anti-CTLA-4 antibody failed to reverse suppression. This observation suggests that in these studies, CTLA-4 was not responsible for the suppressive phenotype of the CD4+CD25+ T cells observed *in vitro*. Indeed the role

of CTLA-4 in CD4+CD25+ T-cell mediated suppression is also somewhat contradictory. Studies have shown that suppression of IBD by $CD45RB^{low}$ cells can be reversed following treatment with a blocking antibody to CTLA-4 (Read *et al.*, 2000). The acceptance of skin grafts mediated by the generation of alloantigen specific CD4+CD25+ T cells can also be reversed following treatment with a blocking anti-CTLA-4 antibody (Kingsley *et al.*, 2002). Interestingly partial reversal of suppression with an antibody to CTLA-4 has also been achieved using human thymocyte-derived CD4+CD25+ T cells and full reversal was achieved with antibodies to both CTLA-4 and TGFβ (Annunziato *et al.*, 2002). Assays conducted using CD4+CD25+CTLA-4+ enriched human regulatory cells have also shown a partial reliance on CTLA-4 for function. However this was not duplicated with CD4+CD25+CTLA-4- cells (Birebent *et al.*, 2004), thus highlighting again the complexity of the CD4+CD25+ T-cell population. *In vitro* studies have also shown that cross-linking of CTLA-4 resulted in the secretion of TGFβ, thus providing another potential mechanism by which CTLA-4 may act (Chen *et al.*, 1998).

However in agreement with the observations presented earlier in this chapter, studies by various groups have shown that blocking CTLA-4 *in vitro* did not result in the reversal of suppression (Thornton and Shevach, 1998; Levings *et al.*, 2001; Baecher-Allan *et al.*, 2001). Also CD4+CD25+ cells from $CTLA\text{-}4^{-/-}$ mice have been shown to possess suppressive activity (Piccirillo and Thornton, 2004). Overall, the likely conclusions are that the role of CTLA-4 *in vitro* is not vital for function but in a more complex *in vivo* environment, it is likely to play a role either *via* the generation of TGFβ or directly by acting on APCs. CTLA-4 may even act directly on the CD4+CD25- effector cell *via* interaction with CD80 or CD86 and initiate reverse

signalling back into the effector cell, thus limiting T cell expansion (Paust *et al.*, 2004).

The addition of exogenous IL-6, TNFα and LT had no effect on the ability of the CD4+CD25+ cells to suppress, which was a surprising result. In a proinflammatory environment one might expect suppression to be reversed, resulting in effective CD4+ helper T cell responses. Indeed a study looking at the functionality of CD4+CD25+ cells from rheumatoid arthritis patients has shown the cells were ineffective at inhibiting cytokine production from CD4+CD25- cells, although they were able to inhibit proliferation. However cells isolated from patients on anti-TNFα therapy showed restoration of CD4+CD25+ function, suggesting that elevated levels of TNFα can directly or indirectly compromise regulatory T cell function (Ehrenstein *et al.*, 2004). Data from Pasare *et al.* showed that IL-6 in combination with another TLR-induced cytokine could also block CD4+CD25+ mediated suppression (Pasare and Medzhitov, 2003). Undoubtedly more experiments need to be done *in vitro*, ideally with combinations of cytokines to fully determine the effect of proinflammatory cytokines on regulatory T cell function. However perhaps it is a misconception that a proinflammatory environment would compromise regulatory T-cell function. Important evidence is now emerging that CD4+CD25+ regulatory T cells may be functional when pathogens invade in order to control the magnitude of the effector T cell response and to prevent excessive tissue damage (Rouse and Suvas, 2004).

The high constitutive expression of CD25 and the observation that $CD25^{-/-}$ mice develop a fatal lymphoproliferative disease (Willerford *et al.*, 1995) led to experiments to investigate whether this molecule was involved functionally in

suppression. Using a blocking antibody to CD25, no effects on suppression were observed. Blocking CD122 or neutralising IL-2 had no effect either. Together these data suggested that IL-2 signalling was not playing a significant role in the generation of suppressor function in these studies. However data from mouse studies showed that transient production of IL-2 by CD4+CD25- responder cells was necessary to drive CD4+CD25+ suppressor cell function (Thornton *et al.*, 2004). Is this highlighting another difference between mouse and human regulatory cells or is this an *in vitro* characteristic of mouse cells that is not replicated *in vivo*? Studies with regulatory cells that had been isolated following homeostatic expansion *in vivo*, showed that although these cells had downregulated CD25 expression, they were still functional suppressors, suggesting that CD25 was not essential for regulatory T cell function (Gavin *et al.*, 2002). Evidence from the studies described in this thesis has also shown that regulatory T cells enriched by selection for TNFR2 expression, had lower levels of CD25 expression than regulatory T cells enriched for CD95 expression, yet the TNFR2+ cells were more potent suppressors. Emerging evidence seems to support the concept that IL-2 –IL-2R signalling is essential in the generation of CD4+CD25+ regulatory T cells but not necessarily the functionality (Malek and Bayer, 2004).

This chapter has highlighted both the complexities and controversies surrounding regulatory T-cell biology. It is clear that there are discrepancies between both *in vitro* and *in vivo* systems and also between human and mouse CD4+CD25+ T cells. Resolution of some of these differences will require the identification of more robust cell surface markers that may result in the sub-typing of different CD4+CD25+ T-cell subsets with different functions. The identification of dysfunctional CD4+CD25+ T cells in human diseases should also aid our understanding of how these cells function.

Perhaps most importantly, the generation of complex human assays that mimic more closely the *in vivo* environment in which many of the mouse studies have been conducted, would also be highly desirable. A new mouse model designed to study human immune cells *in vivo* has recently been described (Traggiai *et al.*, 2004). The RAG2-/-γc-/- mouse is engrafted with human CD34+ stem cells from cord blood and eight to twelve weeks after engraftment, self-renewing human leukocytes can be detected including CD4+CD25+ regulatory T cells. Maybe in the future this model can be adapted to investigate the biology of human CD4+CD25+ cells *in vivo*. This would allow some of the complex interactions between both the innate and adaptive immune response to be followed and together this might help answer some of the currently unresolved questions surrounding regulatory T cell biology.

Chapter 4

Cloning of Human *FOXP3*

and

Optimisation of Transfection Conditions.

4.1 Introduction

In chapter 3 it was confirmed that freshly isolated human CD4+CD25+ T cells had the ability to suppress proliferation of CD4+CD25- responder T cells, following T cell activation. An attempt to identify more specific cell surface markers for this regulatory population of T cells proved unsuccessful. However in 2003, three papers were published identifying the existence of a new, more specific intracellular marker for CD4+CD25+ regulatory cells (Hori *et al.*, 2003; Khattri *et al.*, 2003; Fontenot *et al.*, 2003). These papers also provided evidence that this molecule, called FOXP3, appeared to play a role in the generation of suppressive activity.

The identification of FOXP3 as a critical regulator of CD4+CD25+ regulatory T cells has generated a lot of interest and today, a significant proportion of the research on regulatory T cells centres on FOXP3. However at the commencement of these studies, the evidence that FOXP3 was highly expressed in CD4+CD25+ regulatory T cells was not in the public domain. Data implicating a role for FOXP3 in regulatory T cell function was also unpublished. Studies published in 2001 had identified a mutated form of the *Foxp3* gene as being responsible for the phenotype of the scurfy mouse (Brunkow *et al.*, 2001). This was supported by data from IPEX patients who also exhibited mutations in the *FOXP3* gene (Smyk-Pearson *et al.*, 2003). Together with data from studies in Jurkat T cells (Schubert *et al.*, 2001) and from studies using cells isolated from *Foxp3* transgenic mice (Khattri *et al.*, 2001), the evidence suggested that FOXP3 played an important role in controlling T cell responsiveness to activation. The link with regulatory T cell function, although not published, had also been made and studies confirming this in the mouse were ongoing. However at this stage, there

was no published data to support the function of this protein in human regulatory T cells.

The initial aim of this chapter was to confirm the expression of FOXP3 in human CD4+CD25+ regulatory T cells and then generate the necessary reagents to investigate the effect of overexpression of FOXP3 in human T cells. This required the cloning of both full-length human *FOXP3* and a mutated from of the gene lacking a functional FKH domain. Validation of an appropriate transfection system was also carried out. The initial strategy employed a GFP-based expression system to allow for selection of transfected cells. Subsequently however the strategy changed and a co-transfection system was validated. This system involved the transfection of two separate plasmids, one encoding *FOXP3* and the second one encoding a chimeric receptor. All chimeric receptor constructs were kindly provided by Dr. Helene Finney.

The chimeric receptor used in the initial validation studies described in this chapter, consisted of the intracellular signalling region of the TCR zeta chain (TCRζ) fused to the intracellular signalling region of CD28 (figure 4.1).

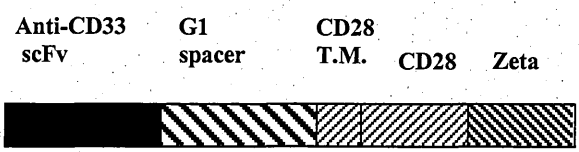


Figure 4.1 Schematic diagram of the CD28/TCRζ chimeric receptor.
The chimeric receptor consists of a single chain Fv specific for CD33, linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3. The spacer is linked to the transmembrane (T.M.) and intracellular regions of human CD28, which is linked to the intracellular region of human TCRζ.

The extracellular region of the receptor is an antibody single chain Fv (scFv) that binds to CD33. It has been reported that the addition of chimeric receptor transfected human T-cells to CD33-coated antigen plates, induces a strong activating signal into the transfected cells through the scFv (Finney *et al.*, 2004). This mechanism of activation ensures that only the transfected cells receive stimulation. Interestingly following transfection of T cells with the dual chimeric receptor, the strength of signal delivered through the chimeric receptor was found to be optimal with the CD28 signalling region membrane proximal and the TCRζ signalling region membrane distal (Finney *et al.*, 1998). This co-transfection system has been adopted for use throughout these studies.

4.2 Results

4.2.1 Expression of FOXP3 in human CD4+CD25+ regulatory T cells

Human CD4+CD25+ and CD4+CD25- T cells were purified from peripheral blood as described in chapter 3. Cell lysates were prepared and assayed for total protein content. Equal amounts of protein were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3. The blots shown in figure 4.2 confirmed that the levels of FOXP3 expression were higher in the CD4+CD25+ T cell population as compared to the CD4+CD25- T cell population. The blots also revealed that FOXP3 appeared to migrate as a doublet with one band of approximately 50kDa corresponding to full-length FOXP3 and a slightly lower molecular weight band. This suggested the possibility that there might be more than one species of FOXP3. Further work investigating the identity of the doublet is described in chapter 6.

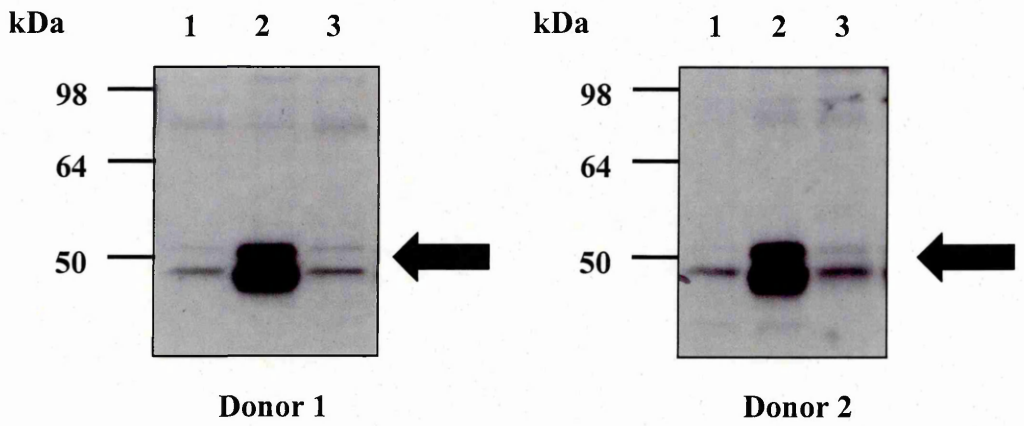


Figure 4.2 Expression of FOXP3 in human CD4+CD25+ and CD4+CD25- T cells.
Cell lysates from human CD4+CD25- (lanes 1 and 3) and CD4+CD25+ (lane 2) T cells were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. The arrow indicates the position of the FOXP3 doublet.

4.2.2 Cloning of human FOXP3

It was decided initially to clone full-length human *FOXP3* into pTracer-CMV2 (figure 4.3) *via Eco*RI and *Not*I restriction sites. This vector was chosen because it contains the GFP gene. Cells that express GFP should also co-express FOXP3, therefore selection of GFP expressing cells provides an efficient method for enrichment of transfected cells.

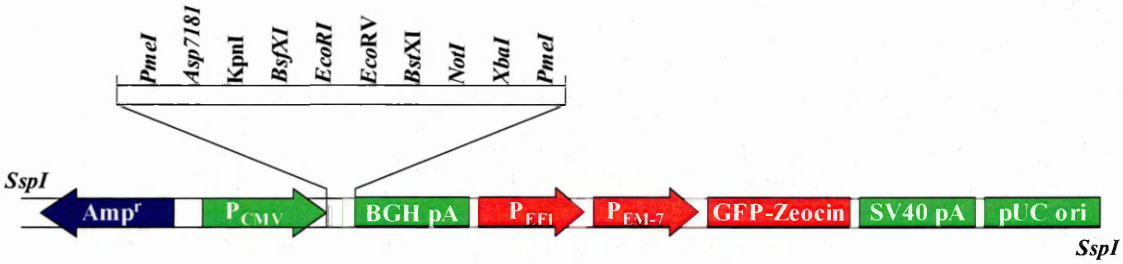


Figure 4.3 Schematic diagram of pTracer-CMV2.
The vector is approximately 6.2kb in size and contains the human cytomegalovirus (CMV) promoter which drives expression of cloned genes. Other essential elements include a multiple cloning site; bovine growth hormone (BGH) polyadenylation signal to allow efficient transcription termination and polyadenylation of cloned mRNA; human elongation factor 1a-subunit promoter (P_{EF1}) to allow mammalian expression of the Cycle 3-GFP-Zeocin™ fusion; a synthetic bacterial promoter, EM-7 (P_{EM-7}) to allow bacterial expression of the Cycle 3-GFP-Zeocin™ fusion; cycle3-GFP, an improved GFP gene fused to zeocin (GFP-zeocin) to detect transfected cells; a zeocin resistance gene for selection in both mammalian cells and *E.coli* and a SV40 polyadenylation signal. The vector also contains the β-lactamase gene to confer resistance to ampicillin and a pUC origin for high copy number replication and propagation in *E.coli.*

The source of cDNA used to clone the FOXP3 gene was human PBMC cDNA. This was provided by Dr. Patrick Slocombe and was generated from a mixture of both resting and activated PBMCs, from a number of different donors. The published sequence (Genbank accession number AF277993) was used to design primers huFOXP3FWD2 (5' primer) and huFOXP3REV (3' primer) to allow amplification of the *FOXP3* gene. The 5' oligonucleotide contained an *Eco*RI site to facilitate cloning and a partial Kozak translational initiation sequence for optimal gene expression, upstream of the initiator methionine. The 3' oligonucleotide contained a stop codon and a *Not*I restriction site. The base composition plot in figure 4.4 shows that the *FOXP3* gene contains certain regions which are very G-C rich. These regions can cause problems during PCR reactions through the formation of secondary structures that resist denaturation and prevent primer annealing. Thus it was decided to include varying concentrations of DMSO in the PCR mix to disrupt the G-C base-pairing. The

polymerase selected for use in the PCR reactions was KOD Hot Start polymerase. This polymerase contains a proof-reading enzyme to minimise PCR errors.

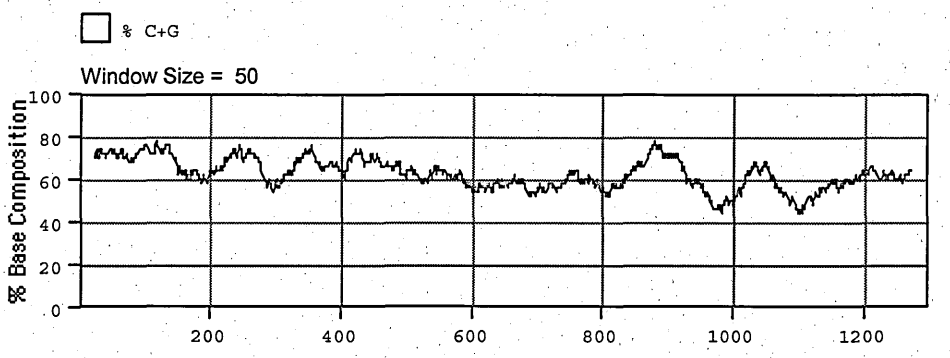


Figure 4.4 G-C base plot for the human *FOXP3* gene.
Analysis of the human *FOXP3* gene highlighting regions where there are a high percentage of G-C base pairings. These G-C rich regions can lead to the formation of secondary structures during the PCR reaction

Six PCR reactions were set-up with increasing amounts of DMSO, ranging from no DMSO to 5% DMSO. Although all the conditions appeared to successfully generate a PCR product of approximately 1.3kb, it was decided to set-up a large scale cloning PCR reaction with 5% DMSO. The large-scale PCR reaction was successful and the PCR product was subjected to a phenol-chloroform extraction to remove contaminants from the PCR reaction. Both the PCR product and the cloning vector (pTracer-CMV2) were then digested with *Eco*RI and *Not*I and following a second phenol-chloroform extraction, the digests were separated on a 1% TAE agarose gel by gel electrophoresis and the bands excised under low wavelength uv light. The gel fragments were purified using a Qiagen gel extraction kit and ligations were set-up overnight at 16°C.

Ligated DNA, both vector alone and vector containing *FOXP3* DNA (insert) were transformed separately into chemically competent *E.coli*. The transformation appeared to be successful with a large stimulation over the vector alone control. Twelve individual colonies were cultured and plasmid DNA was purified. To identify which clones contained the full-length *FOXP3* gene, diagnostic digests were carried out using the restriction enzymes, *Eco*RI and *Not*I. The reactions were then separated by gel electrophoresis on a 1% TAE gel. Figure 4.5 shows that digestion of the clones yielded insert fragments of three different sizes. The expected fragment size was approximately 1.3kb, corresponding to the full-length *FOXP3* gene, yet there appeared to be two smaller fragments. It was decided to sequence clones 2, 4, 6 and 10 and compare the sequence with the published sequence for human *FOXP3* (figure 4.6).

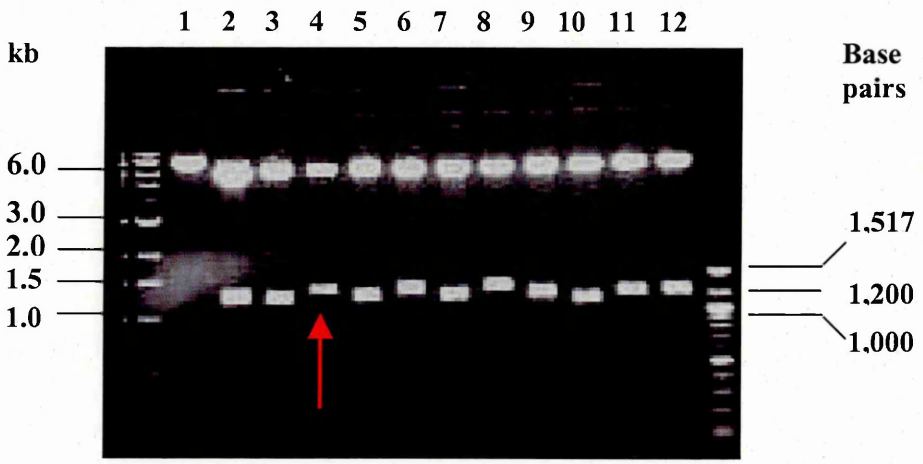


Figure 4.5 Identification of pTracer-CMV2 plasmids containing the full-length *FOXP3* gene.
Small-scale DNA preps were made of 12 clones. Diagnostic digests were carried out on each clone using the restriction enzymes *Eco*RI and *Not*I. Fragments were separated by gel electrophoresis on a 1% TAE gel. The red arrow indicates the fragment corresponding to full-length *FOXP3*.

The sequence alignment data confirmed that whilst clone 4 (and clone 6, not shown) contained full-length *FOXP3*, clone 2 (mutant 1) had a missing region of 105 bases and clone 10 (mutant 2) whilst also missing this 105 base region, had an additional missing region of 81 bases. At this stage of the study the attention centred on the full-length clone, clone 4. Studies investigating clones 2 and 10 are described in chapter 6.

*Eco*RI

5'- GAGAGAGAATTCGCCACCATGCCCAACCCTAGGCCAGCCAAG - 3'

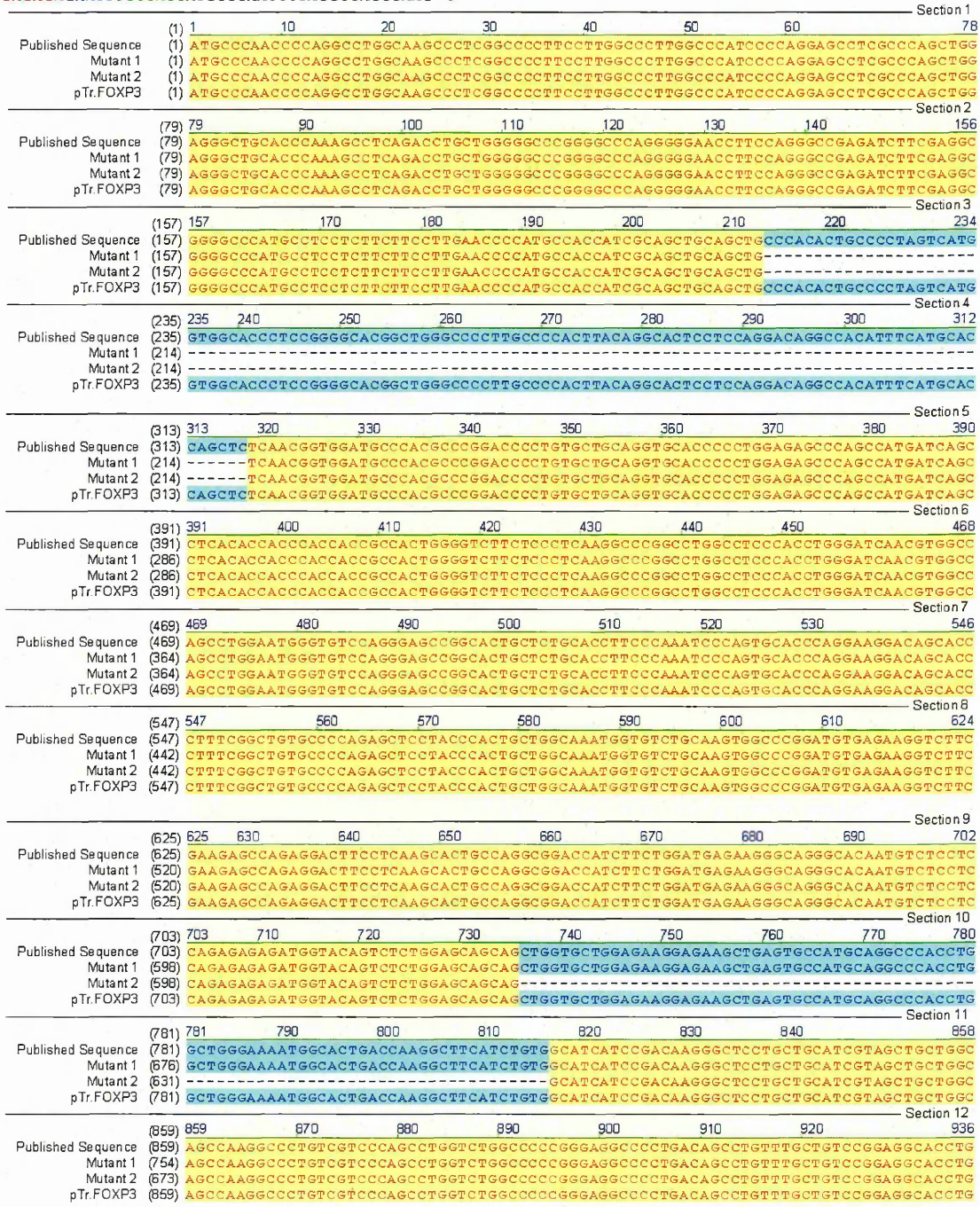


Figure 4.6 continued on next page

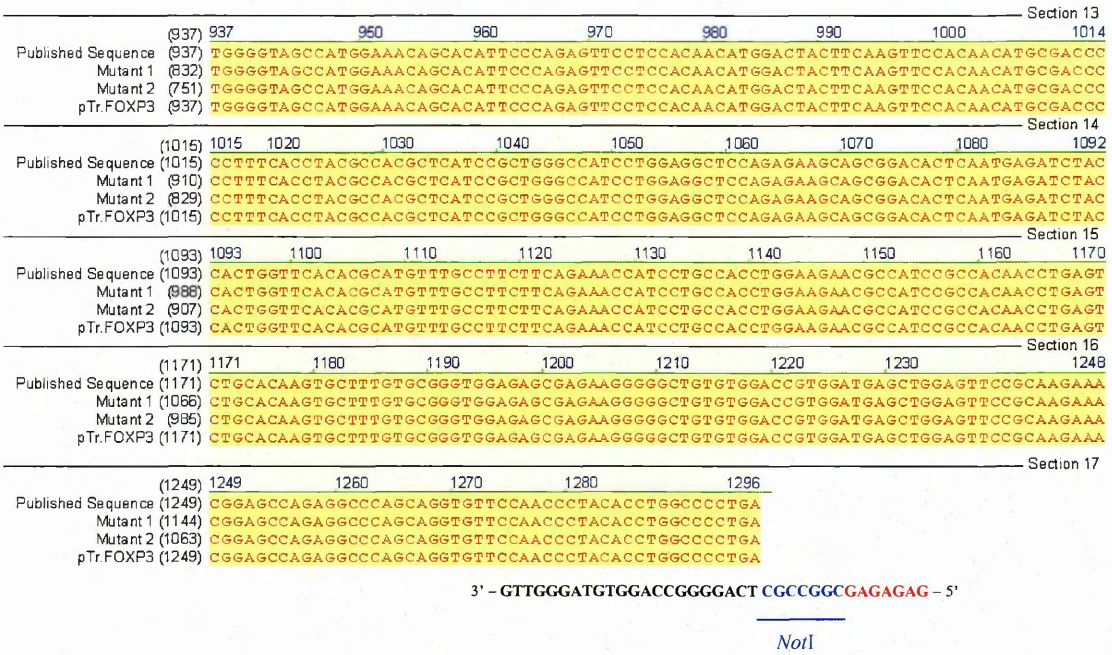


Figure 4.6 Alignment of the published sequence for human *FOXP3* with three different sized clones obtained from PCR reactions using human PBMC cDNA.
Amplification of the human *FOXP3* gene yielded 3 different sized fragments. pTr*FOXP3* (clone 4) corresponds to the full-length 1296bp *FOXP3* gene. Mutant 1 (clone 2) is lacking a region of 105 bases and mutant 2 (clone 10) is missing a region of 186 bases. The 5' and 3' cloning primers are shown. The blue residues indicate the enzyme restriction site and the green residues represent a partial Kozak translation initiation site. The red nucleotides protect the 5'-end of the primer.

4.2.3 Expression of pTracer-CMV2-FOXP3 (pTr-FOXP3)

It was decided that all transfections would be carried out using electroporation. In 2001 Amaxa Biosystems launched its nucleofector™ technology. This technology provides optimised solutions and electrical parameters to allow the transfection of both resting and activated primary cells by electroporation. Previous studies had already validated this methodology for expression of proteins in resting CD4+ T cells (Finney *et al.*, 2004). A large-scale sterile DNA preparation of pTr-*FOXP3* was prepared. To ensure the vector was functional, initial studies involved transfection of DNA into Chinese hamster ovary (CHO) cells. It was decided to transfect 5μg of DNA per $2x10^6$ cells and then analyse the cells by flow cytometry, 24 hours post-transfection. The dot plots in figure 4.7 confirmed that GFP was being expressed following transfection of the pTr.*FOXP3* plasmid. However, attempts to detect intracellular FOXP3 using the polyclonal anti-sera and flow cytometry were unsuccessful (data not shown).

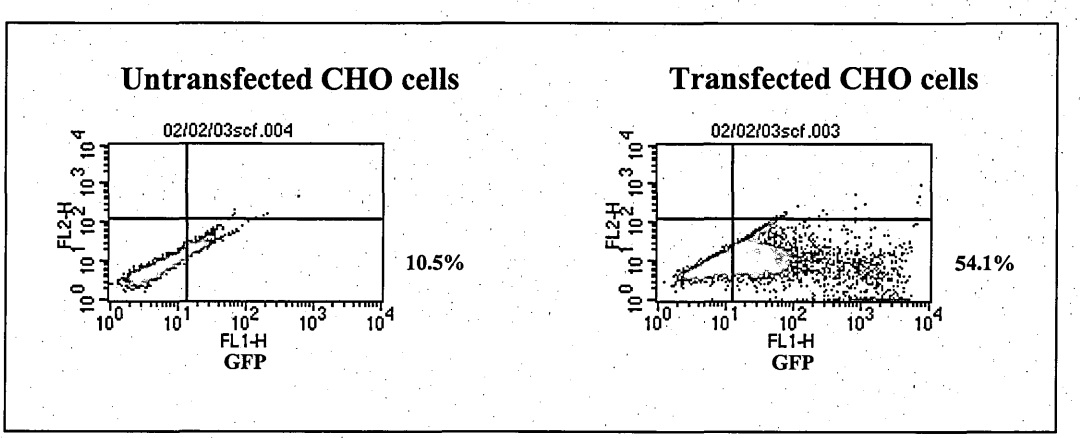


Figure 4.7 Expression of GFP in transfected CHO cells.
CHO cells ($2x10^6$ per transfection) were transiently transfected with 5μg of pTr.*FOXP3* plasmid and analysed for GFP expression by flow cytometry, 24 hours post-transfection. Untransfected cells are shown as a control. The percentage of cells positive for GFP is displayed. Cells likely to be non-viable were excluded on FSC/SSC plots.

Optimisation of expression of GFP in resting human CD4+ T cells was the next goal. Human CD4+ T cells were purified from peripheral blood using a MACS CD4+ negative selection isolation kit as previously described in chapter 3. For the initial experiments, 5μg and 10μg of DNA were transfected per $2x10^6$ CD4+ T cells. As a control, pTracer-CMV2 (pTracer) plasmid without insert was included. Disappointingly the expression levels were very poor with almost no detectable GFP when 5μg of DNA was transfected (data not shown) and only 24% of the cells positive for GFP following transfection with 10μg of pTr-FOXP3 DNA (figure 4.8). Interestingly the percentage of cells positive for GFP expression following transfection with pTracer was 46%. The viabilities of both groups of transfected cells, as determined by forward and side scatter, were also very low with on average, only 35% viability (data not shown).

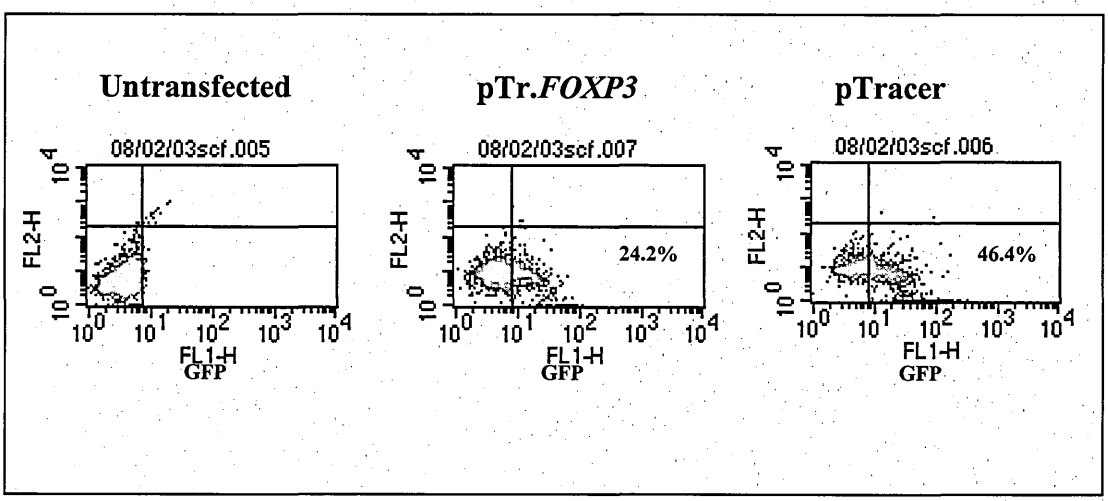


Figure 4.8 Expression of GFP in transfected human CD4+ T cells (I).
CD4+ T cells purified from human peripheral blood using a MACS CD4+ negative isolation kit, were transiently transfected with either 10μg of empty pTracer or 10μg of pTr.*FOXP3* plasmid DNA. $2x10^6$ CD4+ T cells were used per transfection. Cells were analysed for GFP expression by flow cytometry, 24 hours post-transfection. The percentage of cells positive for GFP is displayed. Cells likely to be non-viable were excluded on FSC/SSC plots.

Due to low viability and poor expression levels, an increased cell number of $5x10^6$ cells per transfection was used to try and improve viability. An increased amount of DNA was also used for the transfection to try and boost the expression levels. The data illustrated in figure 4.9 shows the results following transfection of 5, 10 and 20μg of both pTracer and pTr.*FOXP3*. The increased cell number did have a beneficial effect on viability with greater than 65% viability being achieved (data not shown). However the expression levels were still disappointing with only 22% of the cells expressing GFP following transfection with 20μg of pTr-*FOXP3*.

It was observed that at all concentrations of transfected DNA, the empty vector control appeared to be expressed at a much higher level than pTr-*FOXP3*. At the highest concentration of DNA transfected this difference was 39% of cells positive for GFP versus 22%. This suggested the possibility that the CD4+ T cells were finding it difficult to co-express GFP and *FOXP3*. Attempts were made to sort both populations of GFP positive cells using a cell sorter and proliferation assays were set-up in the presence of PHA-L and IL-2. However, due to the low numbers of positive cells, very few cells were recovered and following activation, there were no differences in the levels of proliferation between pTracer and pTr-*FOXP3* (data not shown).

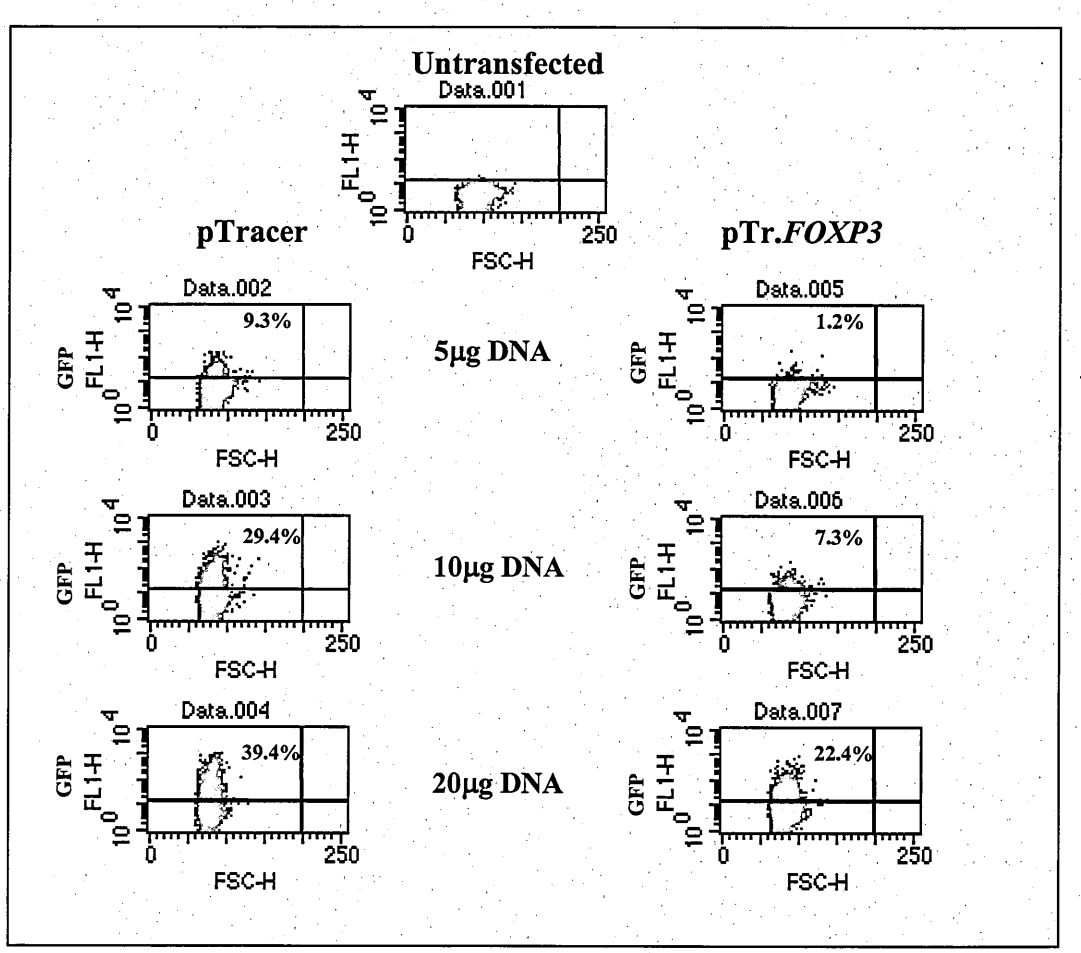


Figure 4.9 Expression of GFP in transfected human CD4+ T cells (II).
CD4+ T cells purified from human peripheral blood using a MACS CD4+ negative isolation kit, were transiently transfected with either 5, 10 or 20µg of empty pTracer or pTr.*FOXP3* plasmid DNA. $5x10^6$ CD4+ T cells were used per transfection. Cells were analysed for GFP expression by flow cytometry, 24 hours post-transfection. The percentage of cells positive for GFP is displayed. Cells likely to be non-viable were excluded on FSC/SSC plots.

4.2.4 Expression of pcDNA3.1.FOXP3 in human CD4+ T cells

Achieving high levels of expression of the pTr.*FOXP3* plasmid in CD4+ T cells had proved difficult. Therefore it was decided at this stage to try a new *FOXP3* plasmid that had been received as a gift from Dr. Roli Khattri. Full-length human *FOXP3* cDNA had been cloned as a *Hin*dIII fragment into the expression vector, pcDNA3.1+ (figure 4.10). One disadvantage of this construct was that it did not contain GFP,

however it did contain a C-terminal flag tag that could be used for detection if required. Initial experiments involved transfecting increasing amounts of both pcDNA3.1 and pcDNA3.1.*FOXP3* DNA into human CD4+ T cells. It was decided to use $4x10^6$ cells per transfection and 6, 9, 12 and 15μg of DNA. The transfected cells were incubated at 37°C overnight, harvested and cell pellets prepared. Cell lysates were then made and assayed for total protein content. Equal amounts of protein were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3.

The immunoblot in figure 4.11 shows that there was a very low level of FOXP3 protein being expressed at 24 hours following the transfection of 6μg of DNA. However transfection of 9μg of DNA resulted in the expression of high levels of FOXP3 protein, producing a distinct band of approximately 50kDa, corresponding to full-length FOXP3 protein. Similar results were observed with 12 and 15μg of DNA.

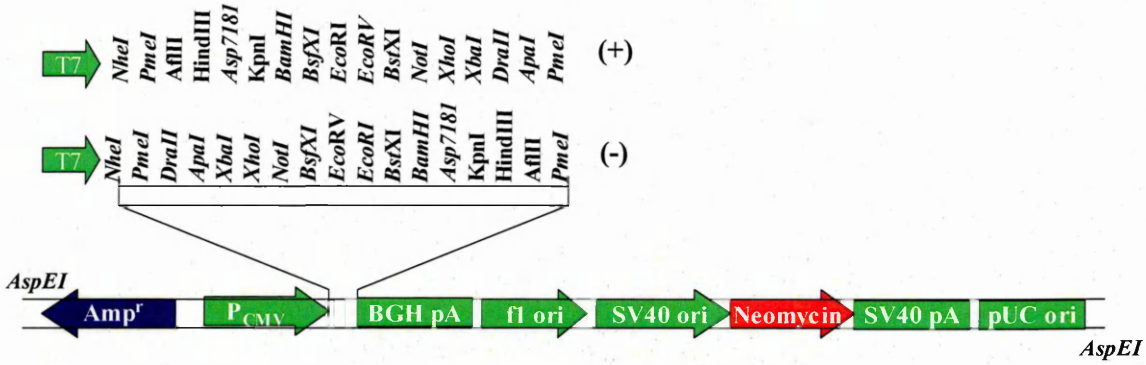


Figure 4.10 Schematic diagram of the expression plasmid, pcDNA3.1+/-.
The vector is approximately 5.4kb in size and contains the human cytomegalovirus (CMV) promoter that drives expression of cloned genes. Other essential elements include a multiple cloning site in both forward and reverse orientations to facilitate cloning; bovine growth hormone (BGH) polyadenylation signal to allow efficient transcription termination and polyadenylation of cloned mRNA; f1` origin of replication; SV40 early promoter and origin of replication to drive high level expression of the neomycin resistance gene, which confers resistance to the antibiotic Geneticin and a SV40 polyadenylation signal. The vector also contains the β-lactamase gene to confer resistance to ampicillin and a pUC origin for high copy number replication and propagation in *E.coli.*

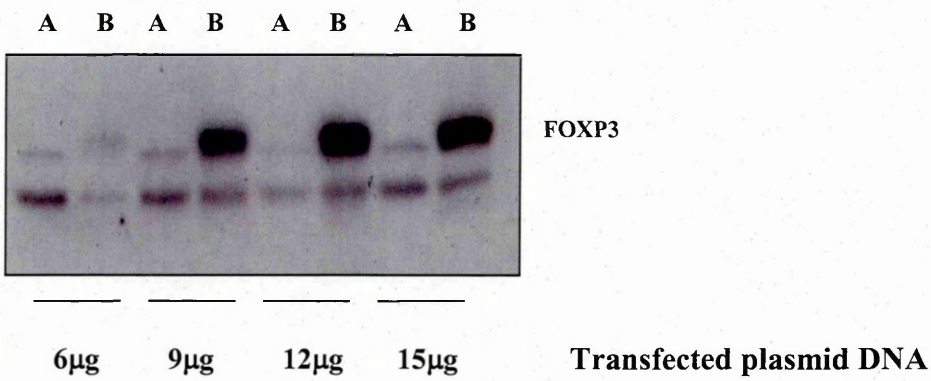


Figure 4.11 Expression of FOXP3 protein in human CD4+ T cells transfected with increasing concentrations of plasmid DNA containing the *FOXP3* gene.
Human CD4+ T cells were purified from peripheral blood using a MACS CD4+ T cell negative selection isolation kit. The CD4+ T cells were transfected with increasing amounts of either empty plasmid DNA (A) or plasmid DNA containing the *FOXP3* gene (B). Cells were harvested (24 hours post-transfection) and cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody.

4.2.5 *Development of a functional assay with CD4+ T cells overexpressing FOXP3*

Transfection of 9μg of plasmid containing *FOXP3* DNA into human CD4+ T cells resulted in easily detectable levels of FOXP3 protein expression. This enabled the studies to progress to the development of functional assays. It was decided initially to investigate the response of transfected cells to activation, using an *in vitro* cell proliferation assay. Purified CD4+ T cells were transfected with either 9μg of pcDNA3.1 or 9μg of pcDNA3.1.*FOXP3*. The transfected cells were rested for 4 hours and then co-cultured with autologous CD4-depleted irradiated PBMCs in the presence of 1μg/ml soluble anti-CD3. Proliferation was determined 3 days later by incorporation of ^{3}H-thymidine. The combined results obtained for 5 individual donors are shown in figure 4.12. Whilst not statistically significant, there was a trend towards reduced levels of proliferation by the cells transfected with *FOXP3* when compared to the vector control transfected cells.

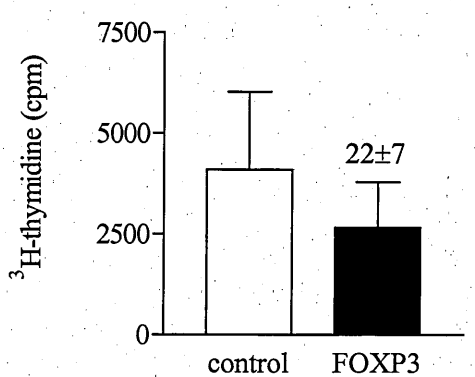


Figure 4.12 Proliferation of CD4+ T cells following transfection with either empty plasmid DNA (control) or plasmid DNA containing the *FOXP3* gene (FOXP3).
Purified human CD4+ T cells were transfected with either empty plasmid DNA or plasmid DNA containing the *FOXP3* gene. 4 hours post-transfection, cells were counted and $2.5x10^{5}$ transfected cells were co-cultured with autologous irradiated CD4-depleted PBMCs ($5x10^{4}$/well) in the presence of 1μg/ml anti-CD3. The proliferative response was measured by ^{3}H-thymidine incorporation during the last 4-6 h of a 3-day culture period. Data shown are mean ± SEM of 5 donors. Value above the bar indicates percentage inhibition ± SEM.

4.2.6 Development of a co-transfection system.

Whilst encouraging, the result in figure 4.12 highlighted some of the limitations of the system. From previous observations with the pTracer construct, it was highly unlikely that the transfection efficiency was 100%. Consequently there were probably a significant proportion of cells within both populations that were not transfected. These cells would be responsive to stimulation by anti-CD3 and could be masking the inhibitory effect of FOXP3 overexpression. The low level of transfection efficiency achieved may also part explain why the FOXP3-transfected cells were not capable of inhibiting the proliferation of the cells likely to be untransfected. A system was therefore required that would ensure activation of only the transfected cells. A co-transfection system was devised in which the *FOXP3* gene was co-transfected with a gene encoding a chimeric receptor. This system allowed activation to be controlled because signalling through the chimeric receptor *via* the scFv, results in the activation of only transfected T cells.

Initial experiments required optimisation of the co-transfection conditions. The blot in figure 4.11 had already shown that transfection of 9μg of DNA appeared to be sufficient for FOXP3 expression. However, because this system also required the co-transfection of chimeric receptor DNA, it was decided to use both 9μg and 12μg of *FOXP3* DNA in combination with 3μg and 6μg of chimeric receptor DNA. Following transfection, the CD4+ T cells were rested for 4 hours and then plated onto CD33-coated 96-well flat-bottomed plates for 24 hours. The scFv portion of the chimeric receptor is specific for CD33, hence binding of CD33 to the scFv portion of the chimeric receptor delivers a strong activating signal into the transfected T cell. In

duplicate, transfected cells were also plated onto uncoated 96-well flat-bottomed plates. Culture supernatants were removed from the CD33 coated plates and assayed for IL-2. The cells from the uncoated plates were removed and analysed for CD33 scFv expression levels by flow cytometry.

The results obtained from the co-transfection experiment were again encouraging. Overexpression of human FOXP3 in human CD4+ T cells appeared to inhibit IL-2 production following specific activation through the chimeric receptor (figure 4.13). This effect although observed with all four different combinations of DNA, appeared to be optimal with 9μg of *FOXP3* DNA and 3μg of CD28/TCRζ chimeric receptor DNA.

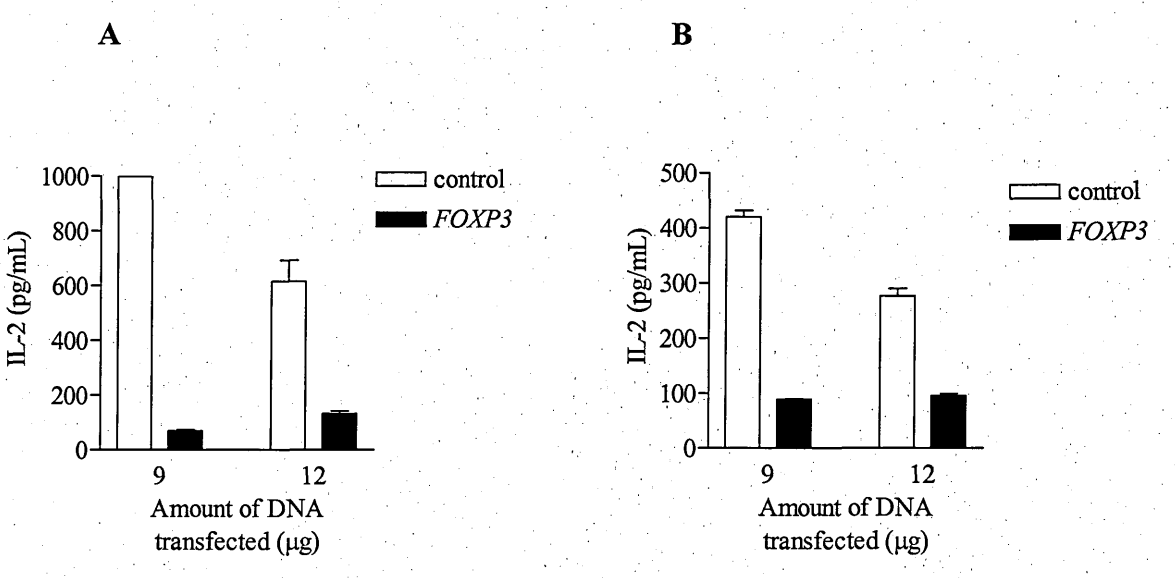


Figure 4.13 Optimisation of co-transfection conditions.
Purified CD4+ T cells were co-transfected with either 3μg (A) or 6μg (B) of CD28/TCRζ chimeric receptor DNA in combination with either 9 or 12μg of *FOXP3* or control plasmid DNA and then rested for 4 hours. Cells were then seeded onto CD33-coated plates ($5x10^5$ cells/well) and incubated for 24 h at 37°C. Supernatants were removed and assayed for IL-2. Data are presented as means of triplicate determinations ± SDs.

The results from the analysis of chimeric receptor expression are shown in figure 4.14. Levels of expression of chimeric receptor appeared to be comparable between the control plasmid transfected cells and the cells transfected with the *FOXP3* plasmid. This result was encouraging as it provided a basis for further studies to investigate the effect of overexpression of FOXP3 in human CD4+ T cells.

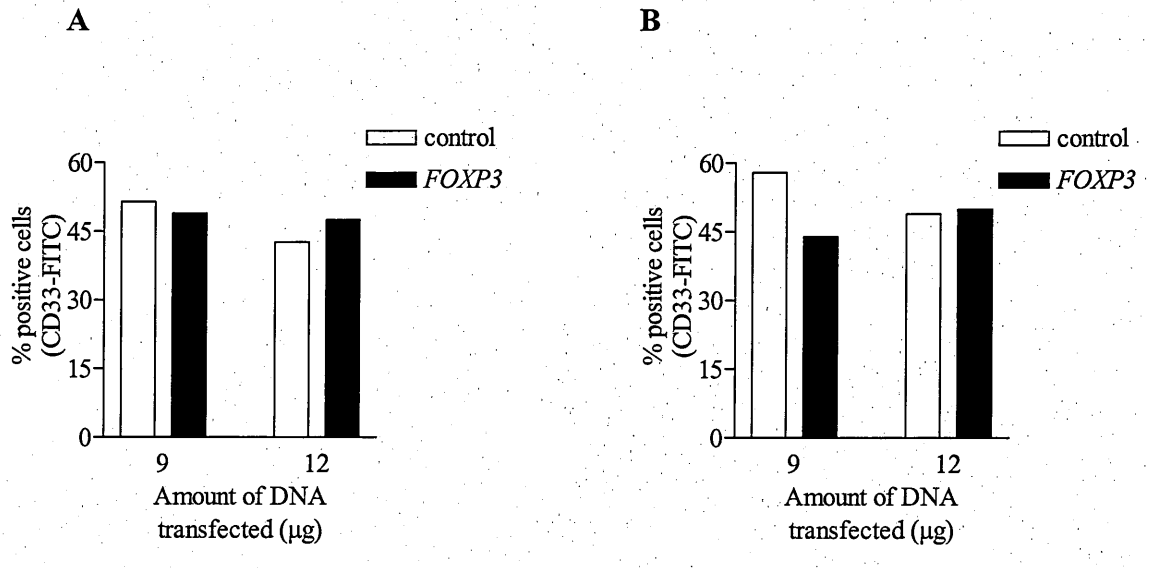


Figure 4.14 Determination of chimeric receptor expression levels following cotransfection of *FOXP3* DNA.
Purified CD4+ T cells were co-transfected with either 3μg (A) or 6μg (B) of CD28/TCRζ chimeric receptor DNA in combination with either 9 or 12μg of *FOXP3* or control plasmid DNA and then rested for 4 hours. Cells were then seeded onto uncoated 96-well flat-bottomed plates ($5x10^5$ cells/well) and incubated for 24 h at 37°C. Cells were removed and analysed by flow cytometry using CD33-FITC to detect the percentage of cells positive for chimeric receptor expression.

4.2.7 Cloning the FKH mutant

The initial observations with *FOXP3* transfected CD4+ T cells had used pTracer-CMV2 without an insert as a control. It was possible that expression of any protein driven by the hCMV promoter would modify T cell behaviour. In order to confirm that the initial observations related to specific effects of FOXP3, a more appropriate control was needed. Based on published data (Schubert *et al.*, 2001) the decision was taken to clone a mutated form of *FOXP3* which encoded a protein that lacked a functional C-terminal FKH domain. The 5' primer that was used to clone the full-length version of the gene was also suitable for cloning the mutant. However a new 3'primer designated FKH_FOXP3_REV was designed. This 3' oligonucleotide contained coding sequence from nucleotide position 981, a flag tag sequence, a terminal stop codon and a *Hin*dIII restriction site. The gene product was amplified from the same human cDNA source that had been used to clone the full-length version, using the same PCR conditions.

The resultant PCR product and the cloning vector, pcDNA3.1- (figure 4.10) were digested with *Eco*RI and *Hin*dIII restriction enzymes. This yielded a PCR product of approximately 1008bp including the flag tag. Both the PCR fragment and the vector were gel-purified and ligations were set-up. Following a successful transformation into *E.coli*, 12 single colonies were cultured and plasmid DNA was purified. Diagnostic digests using *Eco*RI and *Hin*dIII restriction enzymes were carried out on the 12 clones and all appeared to contain an insert of the correct size (figure 4.15). Sequence analysis of clones 1,2 and 3 confirmed the sequence was correct. Figure 4.16 shows the sequence alignment of the FKH clone, *FOXP3* from pcDNA3.1+ and

the published full-length sequence. There is a base change at position 744 in the FKH mutant however this change does not alter the amino acid as both CTG and CTA encode leucine. This change may be the result of a PCR error, although this is thought unlikely and instead it may represent a natural polymorphism. A large-scale DNA preparation was made of the FKH mutant for use as a negative control in the co-transfection experiments.

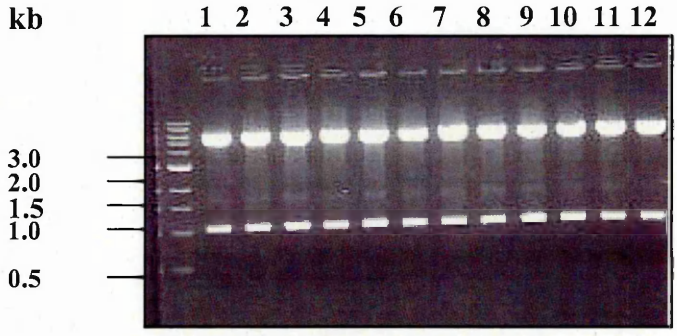


Figure 4.15 Identification of plasmids containing the FKH mutant gene.
Small-scale DNA preps were made of 12 clones. Diagnostic digests were carried out on each clone using the restriction enzymes *Eco*RI and *Hin*dIII. Fragments were separated by gel electrophoresis on a 1% TAE gel. The expected fragment size is approx 1kb.

*Eco*RI

5'- GAGAGAGAATTCGCCACCATGCCCAACCCTAGGCCAGCCAAG - 3'

Section 1

	(1)	1 10 20 30 40 50 60 78
Published Sequence	(1)	ATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCCTTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGG
FOXP3.Flag	(1)	ATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCCTTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGG
FKH.Flag	(1)	ATGCCCAACCCCAGGCCTGGCAAGCCCTCGGCCCCTTCCTTGGCCCTTGGCCCATCCCCAGGAGCCTCGCCCAGCTGG

Section 2

	(79)	79 90 100 110 120 130 140 156
Published Sequence	(79)	AGGGCTGCACCCAAAGCCTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGGC
FOXP3.Flag	(79)	AGGGCTGCACCCAAAGCCTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGGC
FKH.Flag	(79)	AGGGCTGCACCCAAAGCCTCAGACCTGCTGGGGGCCCGGGGCCCAGGGGGAACCTTCCAGGGCCGAGATCTTCGAGGC

Section 3

	(157)	157 170 180 190 200 210 220 234
Published Sequence	(157)	GGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTGCCCACACTGCCCCTAGTCATG
FOXP3.Flag	(157)	GGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTGCCCACACTGCCCCTAGTCATG
FKH.Flag	(157)	GGGGCCCATGCCTCCTCTTCTTCCTTGAACCCCATGCCACCATCGCAGCTGCAGCTGCCCACACTGCCCCTAGTCATG

Section 4

	(235)	235 240 250 260 270 280 290 300 312
Published Sequence	(235)	GTGGCACCCTCCGGGGCACGGCTGGGCCCCTTGCCCCACTTACAGGCACTCCTCCAGGACAGGCCACATTTCATGCAC
FOXP3.Flag	(235)	GTGGCACCCTCCGGGGCACGGCTGGGCCCCTTGCCCCACTTACAGGCACTCCTCCAGGACAGGCCACATTTCATGCAC
FKH.Flag	(235)	GTGGCACCCTCCGGGGCACGGCTGGGCCCCTTGCCCCACTTACAGGCACTCCTCCAGGACAGGCCACATTTCATGCAC

Section 5

	(313)	313 320 330 340 350 360 370 380 390
Published Sequence	(313)	CAGCTCTCAACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAGCCATGATCAGC
FOXP3.Flag	(313)	CAGCTCTCAACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAGCCATGATCAGC
FKH.Flag	(313)	CAGCTCTCAACGGTGGATGCCCACGCCCGGACCCCTGTGCTGCAGGTGCACCCCCTGGAGAGCCCAGCCATGATCAGC

Section 6

	(391)	391 400 410 420 430 440 450 468
Published Sequence	(391)	CTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCCTGGCCTCCCACCTGGGATCAACGTGGCC
FOXP3.Flag	(391)	CTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCCTGGCCTCCCACCTGGGATCAACGTGGCC
FKH.Flag	(391)	CTCACACCACCCACCACCGCCACTGGGGTCTTCTCCCTCAAGGCCCGGCCTGGCCTCCCACCTGGGATCAACGTGGCC

Section 7

	(469)	469 480 490 500 510 520 530 546
Published Sequence	(469)	AGCCTGGAATGGGTGTCCAGGGAGCCGGCACTGCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACC
FOXP3.Flag	(469)	AGCCTGGAATGGGTGTCCAGGGAGCCGGCACTGCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACC
FKH.Flag	(469)	AGCCTGGAATGGGTGTCCAGGGAGCCGGCACTGCTCTGCACCTTCCCAAATCCCAGTGCACCCAGGAAGGACAGCACC

Section 8

	(547)	547 560 570 580 590 600 610 624
Published Sequence	(547)	CTTTCGGCTGTGCCCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGGTCTTC
FOXP3.Flag	(547)	CTTTCGGCTGTGCCCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGGTCTTC
FKH.Flag	(547)	CTTTCGGCTGTGCCCCAGAGCTCCTACCCACTGCTGGCAAATGGTGTCTGCAAGTGGCCCGGATGTGAGAAGGTCTTC

Section 9

	(625)	625 630 640 650 660 670 680 690 702
Published Sequence	(625)	GAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGAAGGGCAGGGCACAATGTCTCCTC
FOXP3.Flag	(625)	GAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGAAGGGCAGGGCACAATGTCTCCTC
FKH.Flag	(625)	GAAGAGCCAGAGGACTTCCTCAAGCACTGCCAGGCGGACCATCTTCTGGATGAGAAGGGCAGGGCACAATGTCTCCTC

Section 10

	(703)	703 710 720 730 740 750 760 770 780
Published Sequence	(703)	CAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGTGCTGGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTG
FOXP3.Flag	(703)	CAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGTGCTGGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTG
FKH.Flag	(703)	CAGAGAGAGATGGTACAGTCTCTGGAGCAGCAGCTGGTGCTAGAGAAGGAGAAGCTGAGTGCCATGCAGGCCCACCTG

Figure 4.16 continued on next page

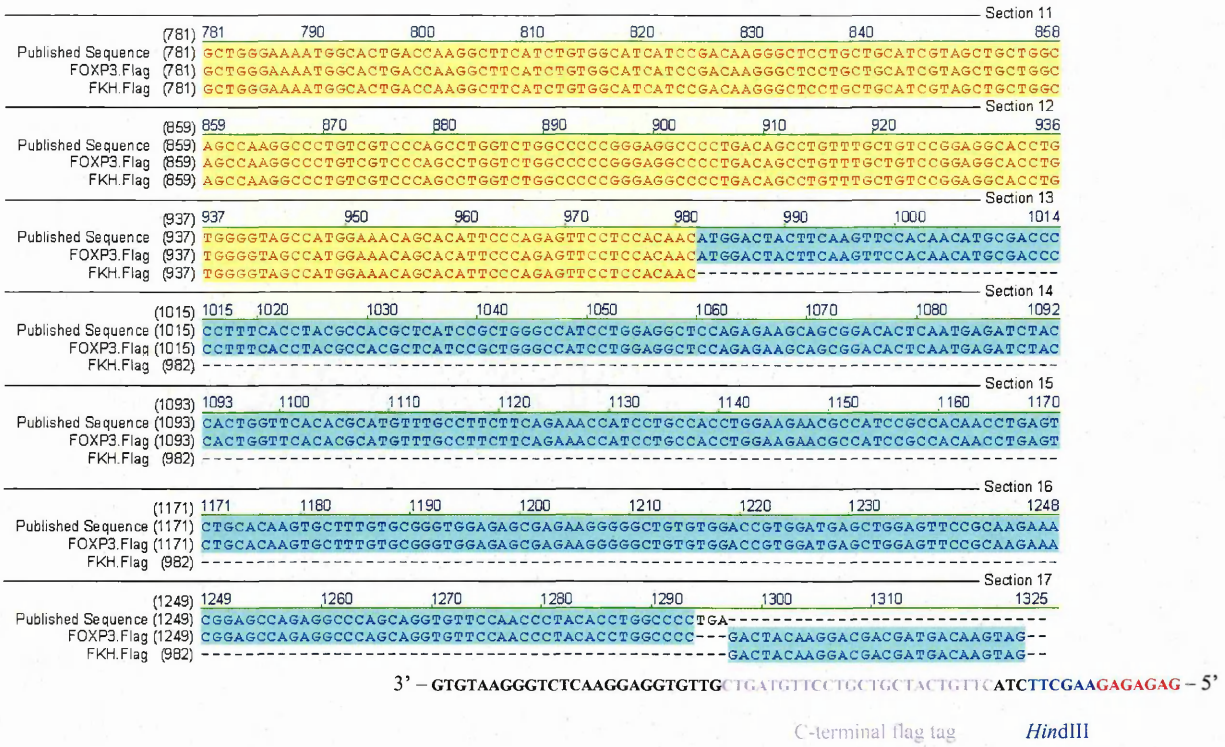


Figure 4.16 Sequence alignment of the published *FOXP3* sequence with the cloned full-length *FOXP3* gene and the FKH mutant.

A mutated form of the *FOXP3* gene lacking a functional FKH domain was cloned from human PBMC cDNA into the expression vector pcDNA3.1. A C-terminal flag tag was included to correspond with the flag tag on the cloned full-length *FOXP3* gene. The 5' and 3' cloning primers are shown. The blue residues indicate the enzyme restriction site and the green residues represent a partial Kozak translation initiation site. The red nucleotides protect the 5'-end of the primer. The purple residues indicate the flag-tag sequence at the C-terminus.

4.3 Discussion

When these studies began, there was no published data linking FOXP3 expression to regulatory T cell function. The phenotype of both the scurfy mouse and IPEX patients suggested a role for FOXP3 in controlling T cell activation. However evidence demonstrating the link with regulatory T cell function was not in the public domain. Studies by Shubert *et al.* had shown that overexpression of FOXP3 in the Jurkat T cell line resulted in inhibition of IL-2 production following stimulation with anti-CD3 (Schubert *et al.*, 2001). This led to the idea that one of the functions of FOXP3 was to act as transcriptional repressor. Studies using CD4+ T cells isolated from transgenic mice overexpressing FOXP3, showed reduced levels of proliferation and IL-2 production following stimulation with anti-CD3 and anti-CD28 (Khattri *et al.*, 2001). This supported the idea that FOXP3 played a role as a regulator of T cell activity.

Prior to the cloning of the *FOXP3* gene, immunoblot studies performed using CD4+CD25+ and CD4+CD25- cell lysates, confirmed that FOXP3 protein was predominantly expressed within the CD25+ population. Interestingly the protein also appeared to be expressed as a doublet. Further investigation into the identity of the doublet is described in chapter 6. There were also faint levels of expression of the protein in the CD25- population. A low level of contaminating CD4+CD25+ T cells within the CD25- population may simply explain this. Alternatively there may be a population of CD25- T cells within the periphery, which express low levels of FOXP3. These cells may act as reservoir of committed regulatory T cells which can be rapidly recruited as required (Yagi *et al.*, 2004). This concept of peripheral

generation of CD4+CD25+ regulatory T cells from CD4+CD25- T cells is further discussed in chapter 6.

In an attempt to try to understand more about the function of FOXP3, the gene was cloned in order to look at the effect of overexpression of FOXP3 in freshly isolated human peripheral blood derived T cells. Historically, transfection of primary cells involved using either retrovirus or adenovirus. However retroviral gene transduction is limited to actively dividing cells as the retrovirus needs to integrate into the host genome. This requires pre-activation of the cells prior to transfection (Miller *et al.*, 1990). Adenovirus can infect resting non-dividing cells but a large volume of virus is required, making this method relatively labour intensive. A new device had recently been validated which delivered DNA directly into the nucleus of primary cells by electroporation. This technique termed nucleofection™, had proved to be more efficient for the transfection of non-activated human CD4+ T cells than the adenoviral method and was quick and relatively easy to use (Finney, 2002).

Transfection of resting cells rather than activated cells was advantageous as it meant that the timing of activation of the T cell and hence the activation of the FOXP3 protein could be controlled. This decision was based on published data showing that the FOXP3 protein is only functionally active following stimulation through the TCR. These published studies have shown that crossing the scurfy mouse with the DO11.10 TCR transgenic strain of mouse on a RAG1 knockout background, produced progeny that were resistant to disease (Zahorsky-Reeves and Wilkinson, 2001). This is because the αβ T cells generated through this cross were strictly reactive only to chicken

ovalbumin peptide, 323-339, therefore in the absence of this peptide, there was no TCR signalling and no activation of the mutated form of FOXP3 protein.

The initial strategy had been to clone the full-length *FOXP3* gene into a GFP-expressing vector and then sort the positive cells on the basis of GFP expression. Transfection efficiencies are never 100% so it was recognised that there would have to be an enrichment step, otherwise the untransfected cells could mask any functional effects of FOXP3 overexpression. Cloning of the *FOXP3* gene into the expression vector pTracer was straightforward. However the percentage of cells expressing high levels of GFP following transfection of the expression vector into both CHO cells and human CD4+ T cells was very poor.

Attempts to improve the expression levels by increasing both the cell number and the amount of DNA transfected, proved ineffective. One possibility was that analysis of GFP expression at 24 hours was too early to detect optimal levels. However analysis of expression levels at 48 hours showed even lower levels of GFP expression (data not shown). The levels of GFP expression were lower following transfection of pTr.*FOXP3* compared to pTracer. One explanation for this observation is that the expression of two intracellular proteins compromised the levels of expression of GFP. The level of FOXP3 expression in these cells was not determined, however it was felt highly unlikely that the FOXP3 protein was directly inhibiting expression of GFP. As already mentioned, for FOXP3 to be functional it requires activation through the TCR and this stimulation was lacking in this system. It was also possible that the pTracer vector was not optimised for use with the nucleofector system.

Efforts switched to a new vector called pcDNA3.1.*FOXP3*. Unlike the pTracer vector, this vector does not contain GFP, hence it was felt expression levels of FOXP3 might be increased. Analysis of FOXP3 expression by immunoblotting showed that FOXP3 protein was being expressed 24 hours post-transfection. However analysis of cell lysates at 48 hours (data not shown) revealed markedly lower levels of protein, indicating that the expression of the protein was very transient. Nevertheless, functional assays set-up in the presence of soluble anti-CD3 indicated a trend towards inhibition of proliferation, following overexpression of FOXP3.

Whilst encouraging, this result also highlighted the need for a system that selected for the transfected cells and a system with an early read-out. The new system that was developed involved cotransfecting pcDNA3.1.*FOXP3* with a chimeric receptor. This system had many advantages over the earlier attempts, primarily because it ensured that only transfected cells could become activated. The fact that two separate vectors were being transfected meant that the expression levels of each could also be optimised. This meant the levels of FOXP3 expression or frequency of cells expressing FOXP3, could be increased without also affecting the levels of chimeric receptor expression. This increased the likelihood that cells transfected with chimeric receptor, were also transfected with FOXP3, whilst reducing the numbers of cells transfected with chimeric receptor alone. The 24-hour timepoint for analysis was also crucial, as previous data had highlighted the transient nature of expression of FOXP3.

The early data validating this system showed that overexpression of FOXP3 appeared to inhibit the production of IL-2 from CD4+ T cells following activation through the chimeric receptor. Levels of expression of chimeric receptor were found to be

equivalent between the control and the *FOXP3* transfected cells. In contrast to the GFP data, the levels of expression of the chimeric receptor were high with over 45% of the viable cells being positive for CD33 scFv expression. These early results provided a novel functional assay system, which could be further developed.

In order to fully exploit this system, it was decided to generate a more appropriate negative control vector. The phenotype of the scurfy mouse is the result of a 2-base pair insertion within exon 8 of the *Foxp3* gene (Brunkow *et al.*, 2001). This mutation results in a frame-shift leading to the generation of a truncated gene product that lacks the carboxy-terminal FKH domain. Genetic studies involving IPEX patients have also shown mutations within the FKH domain (Bennett *et al.*, 2001; Wildin *et al.*, 2001; Wildin *et al.*, 2002). The FKH domain is encoded by sequence covering exons 9, 10 and 11 (Gambineri *et al.*, 2003) and spans amino acid residues 337-420 (Brunkow *et al.*, 2001). Just upstream of the start of the FKH sequence is a potential nuclear localisation signal, starting at nucleotide position 994 (Brunkow *et al.*, 2001). Based on this information and also data from studies using a human FKH mutant (Schubert *et al.*, 2001), a mutated form of the gene that ended at nucleotide position 981 (amino acid 327) was cloned ready for use in expression studies.

These studies have concentrated on the development of a functional co-transfection system to study the effects of FOXP3 overexpression in human T cells. The following chapter uses this system to more fully investigate the effect of FOXP3 overexpression in both human CD4+ and CD8+ T cells. The use of different chimeric receptor constructs is also investigated.

Chapter 5

Overexpression of FOXP3 in Human CD4+ and CD8+ T cells.

5.1 Introduction

Validation of the chimeric receptor co-transfection system in the previous chapter allowed further studies to be undertaken investigating the effect of overexpression of FOXP3 in human T cells. Evidence that FOXP3 plays an important role in controlling T cell activation arose initially from data generated from studies with CD4+ T cells, isolated from both the scurfy mouse and mice transgenic for the *Foxp3* gene. The scurfy mouse mutant has a frameshift mutation within the *Foxp3* gene resulting in a truncated protein lacking a functional FKH domain. Functional analysis of CD4+ T cells isolated from mice with this mutation showed that the CD4+ T cells were hyperresponsive to stimulation (Clark *et al.*, 1999). Cytokine expression was also dysregulated within these mice with elevated levels of many different cytokines being measured (Kanangat *et al.*, 1996). The effects of this mutation are so severe that the mice succumb to a fatal lymphoproliferative disease within a few weeks of birth (Godfrey *et al.*, 1991).

Conversely, characterisation of CD4+ T cells from mice transgenic for the *Foxp3* gene showed that the CD4+ T cells exhibited diminished levels of proliferation and IL-2 production following stimulation, when compared to control animals (Khattri *et al.*, 2001). Together these studies provide evidence to support a role for the FOXP3 protein in controlling T cell activation. This mode of action for FOXP3 is further supported by data generated from studies looking at the effect of overexpression of the human *FOXP3* gene in the Jurkat T cell line. In these studies, overexpression of FOXP3 resulted in an attenuation of activation-induced proliferation and cytokine production (Schubert *et al.*, 2001).

Whilst the studies described in this thesis were progressing, a new mode of action for FOXP3 was proposed. Three seminal papers published within a few weeks of one another showed the selective expression of FOXP3 in mouse CD4+CD25+ regulatory T cells. They also reported that the overexpression of FOXP3 protein in non-regulatory mouse T cells resulted in the acquisition of regulatory activity. This was demonstrated using both cells isolated from mice transgenic for the *Foxp3* gene (Khattri *et al.*, 2003) and cells retrovirally-transduced with the *Foxp3* gene (Hori *et al.*, 2003; Fontenot *et al.*, 2003). Perhaps even more remarkable, was the observation that the retrovirally-transduced cells were able to prevent colitis induced by CD4+CD25-CD45RB^{hi} T cells (Hori *et al.*, 2003). The FOXP3-overexpressing CD4+ T cells also appeared to display a typical mouse CD4+CD25+ regulatory T cell phenotype with elevated expression levels of CD25 and GITR (Hori *et al.*, 2003; Khattri *et al.*, 2003).

At this stage there was no published data confirming this exciting new function for FOXP3 in human T cells, although a paper had been published confirming that FOXP3 expression was associated with human CD4+CD25+ regulatory cells (Walker *et al.*, 2003a). Further experiments by this group had shown that FOXP3 could be upregulated in a proportion of human CD4+CD25- T cells following activation, and that these cells exhibited suppressive activity. These results suggested that FOXP3 expression in human CD4+ T cells, could be directly linked to suppressive activity.

The initial aim of the work described in this chapter was to determine whether the reported role for FOXP3 as a key regulator of T cell activation, as shown by overexpression of the protein in both mouse and Jurkat T cells, was also true for

human peripheral blood derived T cells. In order to determine this, co-transfection experiments were initially carried out using FOXP3 and the CD28/TCRζ chimeric receptor. The ability of FOXP3 to inhibit signalling through the chimeric receptor was measured. Subsequently the effect of FOXP3 overexpression on signalling through different costimulatory pathways was also investigated, using chimeric receptors containing the intracellular signaling regions of ICOS, CD134 and CD137. Finally attention focussed on determining whether the overexpression of FOXP3 in human peripheral blood-derived CD4+ T cells resulted in the generation of suppressive activity.

5.2 Results

5.2.1 Expression of full-length FOXP3 and the FKH mutant.

In the previous chapter, a mutated form of the human *FOXP3* gene was cloned which lacked a functional FKH domain. It was felt that this construct was a more appropriate negative control for functional studies than a control plasmid without an insert. It was important to confirm expression of the FKH mutant in human CD4+ T cells before functional studies were initiated. It was also important to show that the levels of expression of the FKH mutant protein were equivalent to that found with the full-length FOXP3 protein. Human CD4+ T cells were purified from peripheral blood as previously described. Transfections were carried out using 9μg of plasmid DNA containing either full-length *FOXP3* or FKH mutant, per $4x10^6$ CD4+ T cells. Cells were incubated at 37°C for 24 hours. Cell lysates were then prepared and assayed for total protein content. Equal amounts of protein from each transfected cell population were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3. The immunoblot in figure 5.1 confirmed that both the FKH mutant and the full-length FOXP3 protein were being expressed and they appeared to be expressed at similar levels. There are also some faint bands on the immunoblot which may represent endogenous FOXP3 within the CD4+ T cell population.

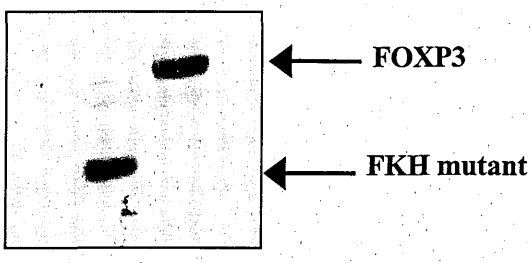


Figure 5.1 Immunoblot of CD4+ T cells transfected with plasmid DNA containing either full-length *FOXP3* or FKH mutant.
Human CD4+ T cells were transfected with plasmid DNA containing either full-length *FOXP3* or FKH mutant. 24 hours post-transfection, cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. The arrows indicate the position of both proteins. Data is representative of 1 of 3 independent experiments.

To further confirm the expression of both forms of the FOXP3 protein, studies were carried out using fluorescence microscopy. Cells were co-transfected with plasmid DNA containing the chimeric receptor and plasmid DNA containing either full-length *FOXP3* or FKH mutant as described before, and incubated for 24 hours without CD33 stimulation. Analysis of transfected cells by flow cytometry includes a gating step to exclude the dead cells. This is either set on the basis of propidium iodide (PI) uptake or FSC/SSC profile. Therefore it was decided to include a dead cell removal step in this procedure to increase the proportion of viable cells and thus aid detection. This was carried out using a dead cell removal kit purchased from Miltenyi Biotec. Cytospins were then prepared and after a fixation step, pre-mixed CD33-FITC and rabbit anti-sera raised against human FOXP3 were added. Following an incubation step, goat anti-rabbit IgG (H+L) Alexa Fluor 660 antibody was added to detect the anti-sera. Slides were then analysed on a Leica fluorescence microscope.

Detection of unactivated human CD4+ T cells was not straightforward as the cells were very small and it was difficult to detect the cytoplasm. Figure 5.2 illustrates some of the results that were obtained. Red staining represents FOXP3 or FKH mutant protein and green staining represents the chimeric receptor protein. Previous immunoblot studies had already confirmed that the FKH mutant protein could be detected by the rabbit polyclonal antisera. Although cells that expressed FOXP3 generally also appeared to express the chimeric receptor, the pattern of FOXP3 expression was different between the FKH mutant (Figure 5.2A) and the full-length FOXP3 protein (Figure 5.2B). Double staining for the chimeric receptor and FKH mutant generated a yellow-orange colour, suggesting that the red and green colours overlie one another. In contrast, distinct red and green staining was observed with the full-length FOXP3 and chimeric receptor co-transfected cells. This suggests that the FKH mutant protein and the full-length FOXP3 protein were localised in different cellular sites within the cell. There was no fluorescence detected when transfected cells were stained with control rabbit serum or when untransfected cells were stained with CD33-FITC (data not shown).

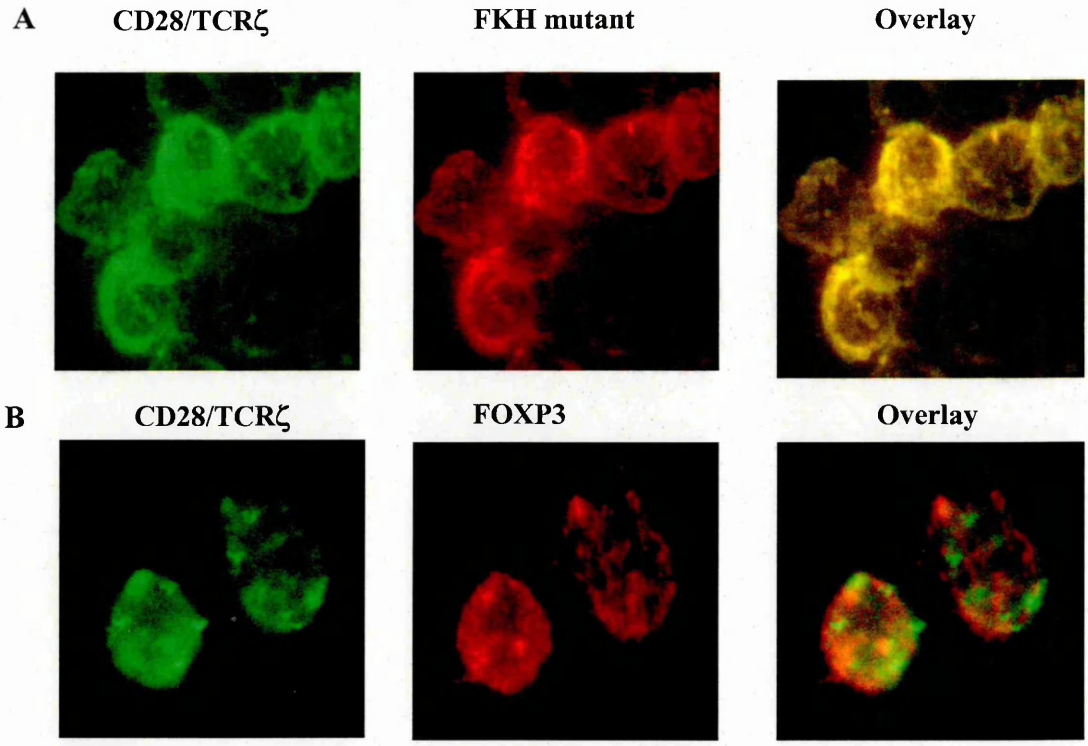


Figure 5.2 Detection of transfected CD4+ T cells by fluorescence microscopy.
Purified human CD4+ T cells were co-transfected with 3μg of CD28/TCRζ chimeric receptor DNA and 9μg of plasmid DNA containing either full-length FOXP3 or FKH mutant. 24 hours post-transfection, dead cells were removed using a MACS dead cell removal kit. Cytospins were prepared and cells were stained with CD33-FITC to detect the chimeric receptor (green) and rabbit anti-sera to FOXP3. Detection of the anti-sera was achieved using a goat anti-rabbit Alexa Fluor 660 antibody (red). Visualisation of the proteins was achieved using a fluorescence microscope.

5.2.2 Investigation into the effect of FOXP3 overexpression on CD4+ T cell activation.

It was initially important to establish that any effects observed following overexpression of full-length FOXP3 were not as a result of lower levels of cell viability, or chimeric receptor expression. Purified CD4+ T cells were co-transfected with 3μg of CD28/TCRζ chimeric receptor DNA and 9μg of either full-length *FOXP3* DNA or FKH mutant DNA. Viabilities were determined 24 hours post-transfection by measurement of FSC/SSC profiles and by the levels of PI uptake. Detection of bound CD33-FITC by flow cytometry indicated the percentage of cells expressing the chimeric receptor.

Figures 5.3A, 5.3B and 5.3C illustrate the data obtained following analysis by flow cytometry of one representative individual donor. Figure 5.3A shows the viability of the co-transfected cells on the basis of FSC and SSC profiles, whilst figure 5.3B shows the viability of the cells on the basis of PI uptake. The levels of chimeric receptor expression are shown in figure 5.3C. The combined results for a number of donors are illustrated in the figures 5.3D and 5.3E. Figure 5.3D shows that the viabilities of the two transfected populations were equivalent with viability levels of around 60% being achieved. Comparison of the PI staining data with the analysis of viability *via* FSC/SSC profiles highlighted no significant differences in the percentage of viable cells. Thus validating both methods as comparable for measuring cell viabilities. There was also no significant difference in the percentage of chimeric receptor positive cells between the two populations of transfected cells with expression levels of around 60% being achieved (figure 5.3E).

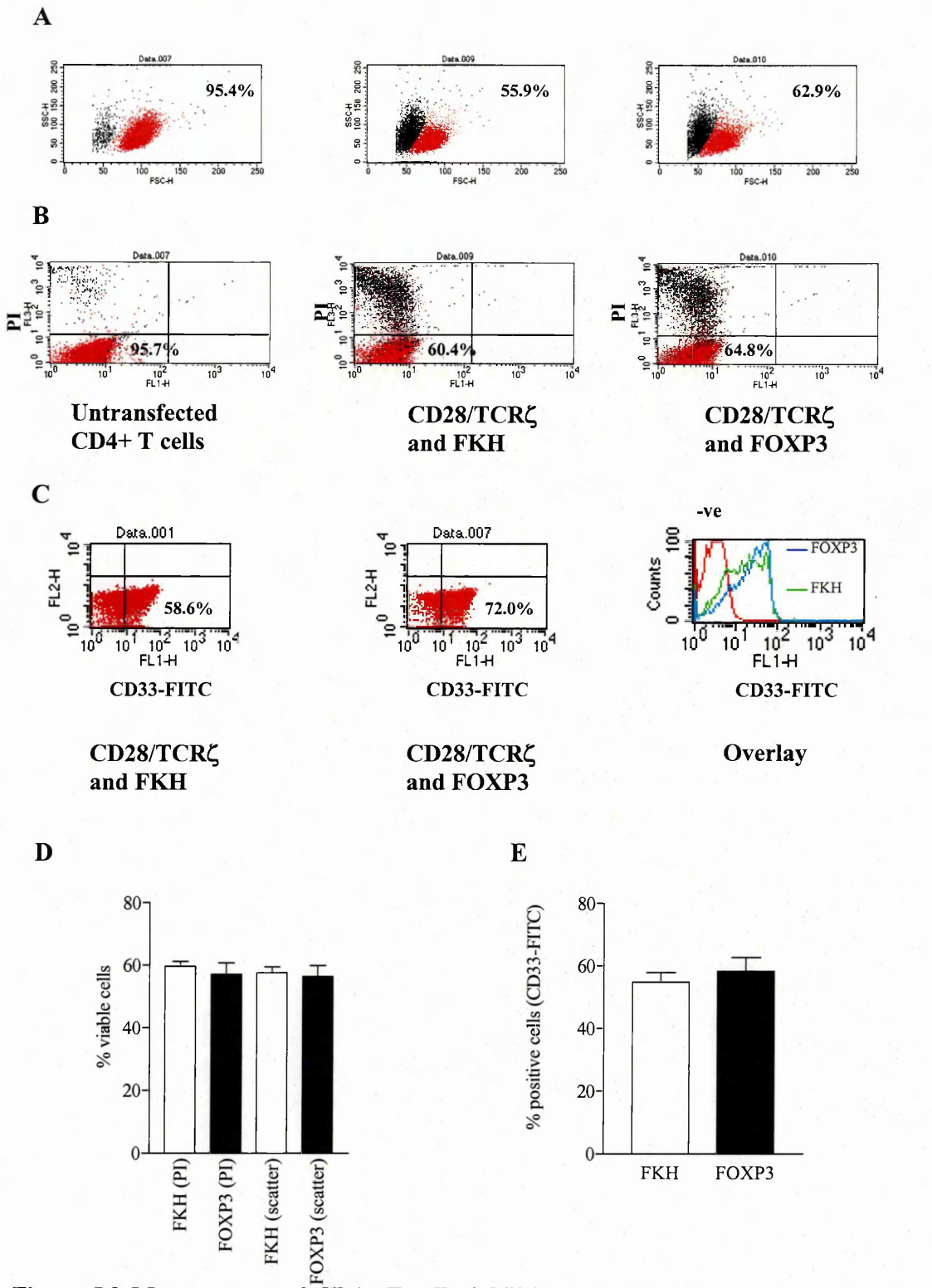


Figure 5.3 Measurement of CD4+ T cell viabilities and chimeric receptor expression levels following co-transfection with *FOXP3* or FKH mutant DNA.

Human CD4+ T cells were co-transfected with CD28/TCRζ chimeric receptor DNA and either DNA encoding FOXP3 or FKH mutant protein. 24 hours post-transfection, cell viabilities were determined using FSC/SSC profiles (A) and PI staining (B). Viable cells are shown in red. Values on dot-plots indicate percentage viable cells. Chimeric receptor expression was measured using CD33-FITC (C), gates were set using untransfected CD4+ T cells (data not shown). Values on dot-plots indicate percentage positive cells expressing chimeric receptor. Combined data for cell viability are mean ± SEM, n=4 (D). Combined data for CD33 expression are mean ± SEM, n=7 (E).

Once it had been established that the transfection efficiency and the viabilities were equivalent between the two transfected populations, subsequent experiments concentrated on investigating whether overexpression of FOXP3 had any functional effect on signalling through the CD28/TCRζ chimeric receptor. Human CD4+ T cells were co-transfected with DNA encoding the CD28/TCRζ chimeric receptor and DNA encoding either FOXP3 or FKH mutant protein and seeded onto CD33-coated plates. The culture supernatants were harvested 24 hours post-transfection and assayed for human IL-2 and human TNFα. Cells were removed and stained for cell surface CD69 and CD25. Figure 5.4A shows that overexpression of FOXP3 in human CD4+ T cells significantly inhibited CD69 upregulation ($p<0.05$) following specific cell activation *via* the CD28/TCRζ chimeric receptor. The levels of CD25 expression on the surface of cells expressing full-length or truncated FKH mutant were not significantly different (Figure 5.4B). However there was also a significant inhibition of IL-2 ($p<0.05$) and TNFα production ($p<0.05$), as compared to the FKH mutant (Figures 5.4C and D). The culture supernatants were also assayed for human IL-10 but the levels were below the limit of detection (assay range is 62.5-4000pg/mL).

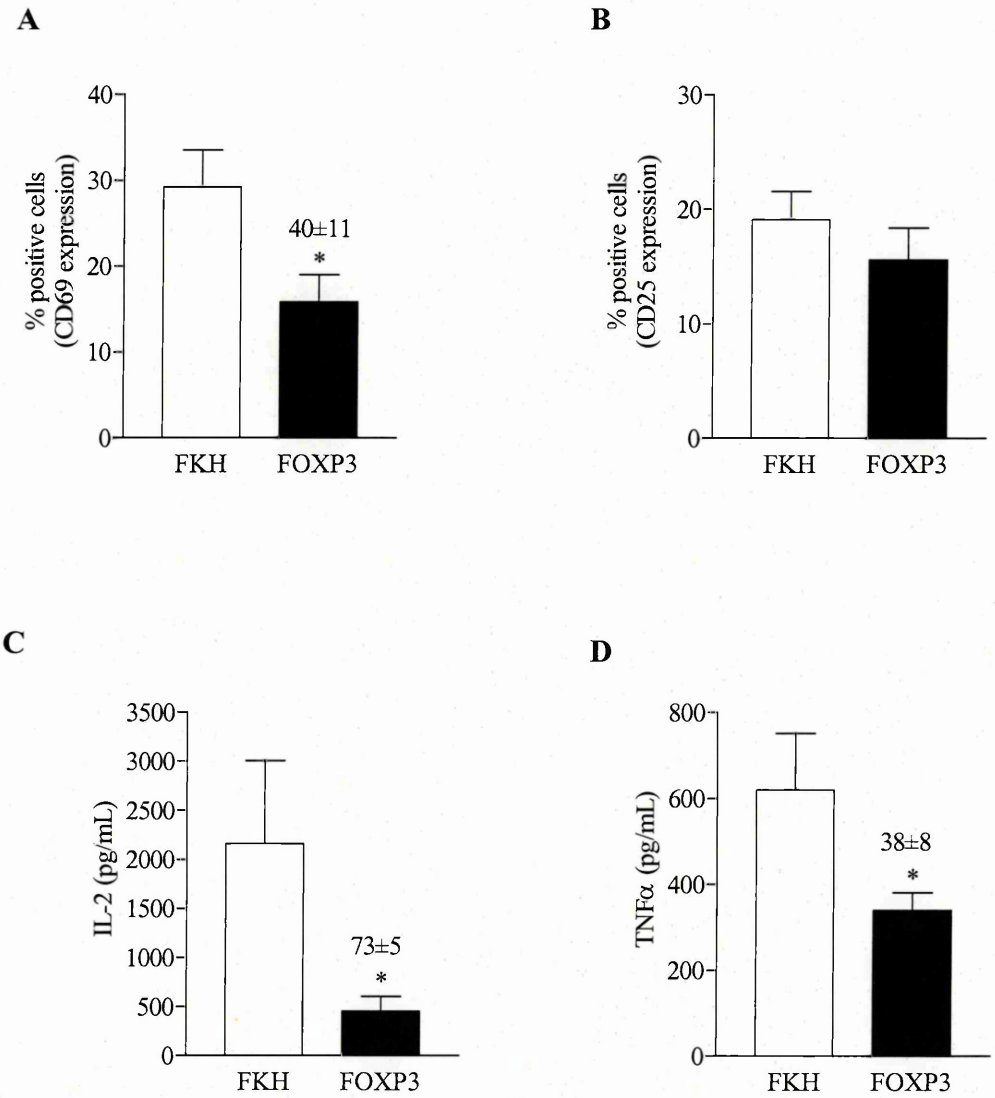


Figure 5.4 Measurement of CD4+ T cell activation following co-transfection of CD28/TCRζ chimeric receptor DNA with either full-length *FOXP3* or FKH mutant DNA.

Human CD4+ T cells were co-transfected with chimeric receptor DNA and either DNA encoding FOXP3 or FKH mutant protein and then rested for 4 hours post-transfection. Cells were then seeded onto CD33-coated plates and incubated for 24 h at 37°C. Cells were removed and stained for CD69 expression levels (A) and CD25 expression levels (B). Supernatants were removed and assayed for IL-2 (C) and TNFα (D). Results are mean ± SEM, n=8. Values above the bars indicate percentage inhibition ± SEM. *, $p<0.05$ as compared with FKH control (Paired T-test).

5.2.3 Investigation into the effect of FOXP3 overexpression on CD8+ T cell activation.

These data have confirmed that FOXP3 can act to inhibit aspects of CD4+ T cell activation. The next set of experiments concentrated on determining whether FOXP3 could also inhibit CD8+ T cell activation. Human CD8+ T cells were purified from peripheral blood using a MACS CD8+ T cell negative selection isolation kit. As for the CD4+ T cells studies, it was important to establish that any effects observed following overexpression of full-length FOXP3, were not as a result of lower levels of cell viability or chimeric receptor expression. Purified CD8+ T cells were co-transfected with 3μg of CD28/TCRζ chimeric receptor DNA and 9μg of either DNA encoding full-length FOXP3 protein or FKH mutant. Viabilities were determined 24 hours post-transfection by measurement of FSC/SSC profiles. Detection of bound CD33-FITC by flow cytometry indicated the percentage of cells expressing the chimeric receptor.

Figure 5.5A shows the viabilities of both populations of transfected cells were equivalent with viability levels of around 50% being achieved. The levels of chimeric receptor expression were also comparable between the two cell populations with expression levels of around 60% (figure 5.5B). Analysis of the expression levels of both full-length FOXP3 and the FKH mutant was carried out by immunoblotting as previously described. Figure 5.6 shows that the expression levels of both proteins appeared to be equivalent. There also appears to be some endogenous FOXP3 being detected in the CD8+ T cell population.

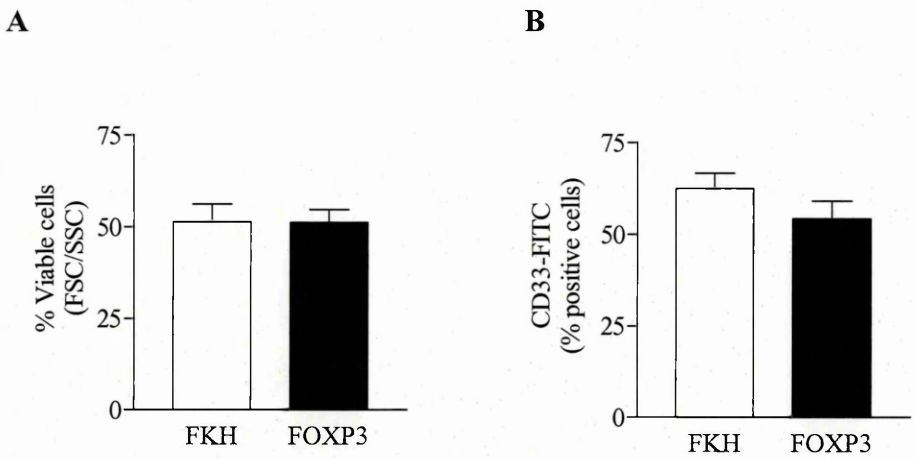


Figure 5.5 Measurement of CD8+ T cell viabilities and chimeric receptor expression levels following co-transfection with *FOXP3* or FKH mutant DNA.
Human CD8+ T cells were co-transfected with chimeric receptor DNA and either DNA encoding FOXP3 or FKH mutant protein. 24 hours post-transfection, cell viabilities were determined by FSC/SSC profiles (A). Chimeric receptor expression was measured using CD33-FITC (B). Results are mean ± SEM, n=4.

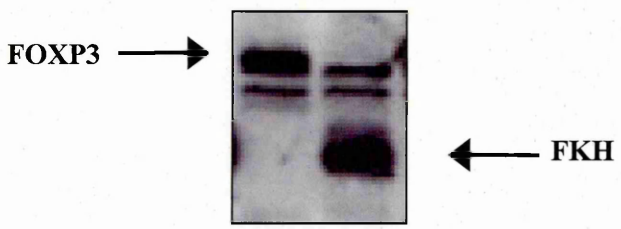


Figure 5.6 Immunoblot of CD8+ T cells transfected with plasmid DNA containing either full-length *FOXP3* or FKH mutant.
Human CD8+ T cells were transfected with plasmid DNA encoding either full-length FOXP3 or FKH mutant protein. 24 hours post-transfection, cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. The arrows indicate the position of both proteins. Data is representative of 1 of 3 independent experiments.

Once the viabilities and expression levels of both the chimeric receptor and the FOXP3 proteins had been confirmed as comparable, attention focussed on investigating whether FOXP3 had any functional effect on CD8+ T cell activation. Human CD8+ T cells were co-transfected with DNA encoding the CD28/TCRζ chimeric receptor and DNA encoding either FOXP3 or FKH mutant protein and seeded onto CD33-coated plates. As for the CD4+ T cell studies, culture supernatants were harvested 24 hours post-transfection and assayed for human IL-2 and human TNFα levels. Cells were also removed and stained for cell surface CD69 and CD25 expression.

In agreement with the data obtained with the human CD4+ T-cells, overexpression of human FOXP3 in human CD8+ T-cells resulted in a significant inhibition of CD69 upregulation ($p<0.001$) and a significant inhibition of IL-2 ($p<0.001$) and TNFα ($p<0.05$) production, as compared to cells overexpressing the FKH mutant (Figures 5.7A, 5.7C and 5.7D). There was trend towards reduced levels of CD25 expression between cells expressing full-length FOXP3 compared to cells expressing the FKH mutant (Figure 5.7B, $p=0.081$). However the levels of CD25 expression on the CD8+ T cells were considerably lower than the levels of CD25 expression on the surface of the transfected CD4+ T cells.

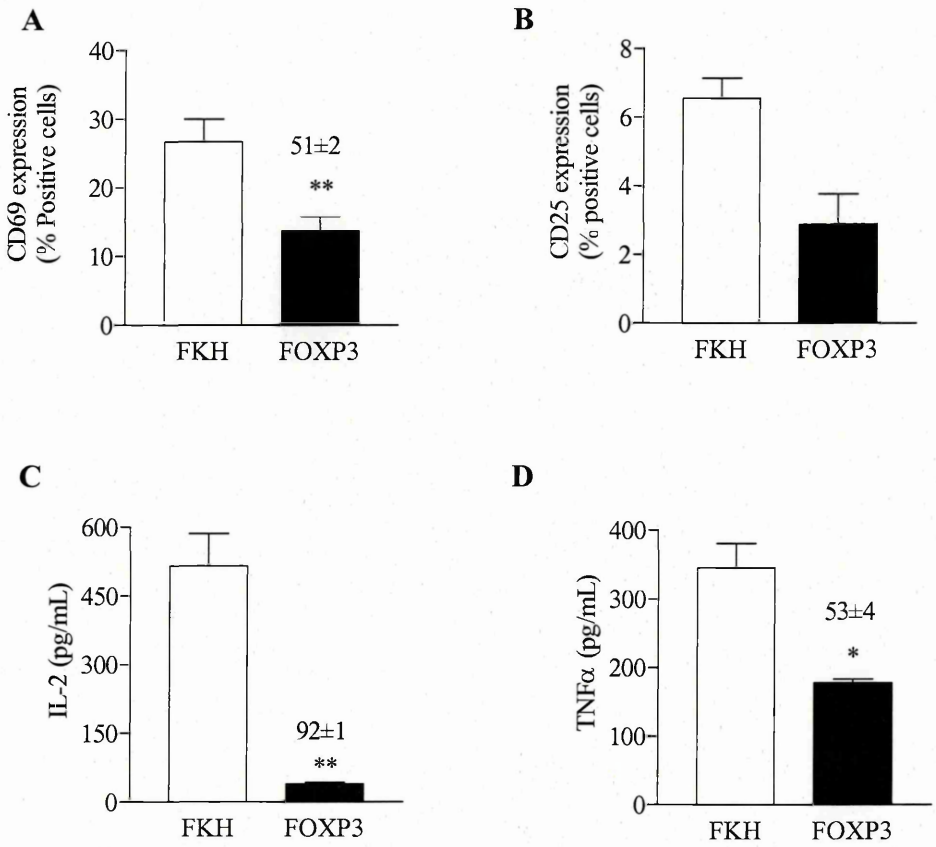


Figure 5.7 Measurement of CD8+ T cell activation following co-transfection of CD28/TCRζ chimeric receptor DNA with either full-length *FOXP3* or FKH mutant DNA.

CD8+ T cells were transfected with CD28/TCRζ chimeric receptor DNA and DNA encoding either FKH mutant or FOXP3 protein. Cells were rested for 4 hours and then seeded onto CD33-coated plates. Cells were removed 24 hours later and stained for CD69 expression levels (A) and CD25 expression levels (B). Supernatants were removed and assayed for IL-2 (C) and TNFα (D). Results are mean ± SEM, n=4. Values above the bars indicate percentage inhibition ± SEM. *, $p<0.05$; **, $p<0.001$ as compared with FKH control (Paired T-test).

5.2.4 Inhibition of IL-2 production is not restricted to the CD28/TCRζ signalling pathway.

These data have shown that overexpression of FOXP3 in both human CD4+ and CD8+ T cells resulted in a significant inhibition of CD28/TCRζ induced T cell activation. It was decided to investigate whether this was also true for other activation pathways. The first of these co-transfection experiments was set-up using a CD134/TCRζ chimeric receptor. This receptor, illustrated in figure 5.8, is identical to the CD28/TCRζ chimeric receptor apart from the intracellular signalling region, which has been replaced with the intracellular signalling region of CD134. Published studies have confirmed the signalling components of this receptor are functional and lead to enhanced levels of IL-2 and TNFα production compared with a chimeric receptor containing the TCRζ signalling region alone (Finney *et al.*, 2004).

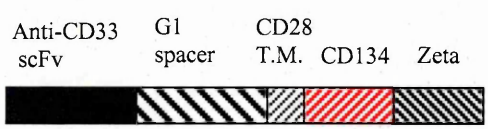


Figure 5.8 Schematic diagram of the CD134/TCRζ chimeric receptor.
The chimeric receptor consists of a single chain Fv specific for CD33, linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3. The spacer is linked to the transmembrane (T.M.) region of human CD28 and the intracellular region of human CD134, which is linked to the intracellular region of human TCRζ.

Human CD4+ T cells were transfected with 3μg of CD134/TCRζ chimeric receptor DNA and 9μg of *FOXP3* or pcDNA3.1 (control vector) DNA. The FKH mutant was not used for these studies because it was unavailable. A proportion of the cells were

then incubated for 24 hours without any further stimulation and stained for cell surface expression of CD134/TCRζ chimeric receptor. As is shown in figure 5.9A, there were comparable levels of expression of the CD134/TCRζ chimeric receptor on both cell populations. The cell viabilities of the two populations, as determined by FSC/SSC profiles, were also equivalent (figure 5.9B).

The remaining cells were seeded onto CD33-coated plates as before and culture supernatants were harvested 24 hours later and assayed for human IL-2 and human TNFα. The cells were also removed and stained for cell surface CD69 and CD25. As is shown in figure 5.10A, overexpression of FOXP3 in human CD4+ T cells resulted in a significant inhibition of CD69 expression ($p<0.001$) following specific cell activation *via* the CD134/TCRζ chimeric receptor. There was also a significant inhibition of IL-2 production ($p<0.05$) as compared to the control vector (Figure 5.10C), although the levels of IL-2 production are considerably lower than those produced following signalling through the CD28/TCRζ chimeric receptor. The levels of CD25 expression and TNFα production were not significantly different between the cells expressing full-length FOXP3 and those transfected with vector alone. (Figures 5.10B and 5.10D). These data show that the ability of FOXP3 to inhibit IL-2 production is independent of the CD28-specific signalling pathway.

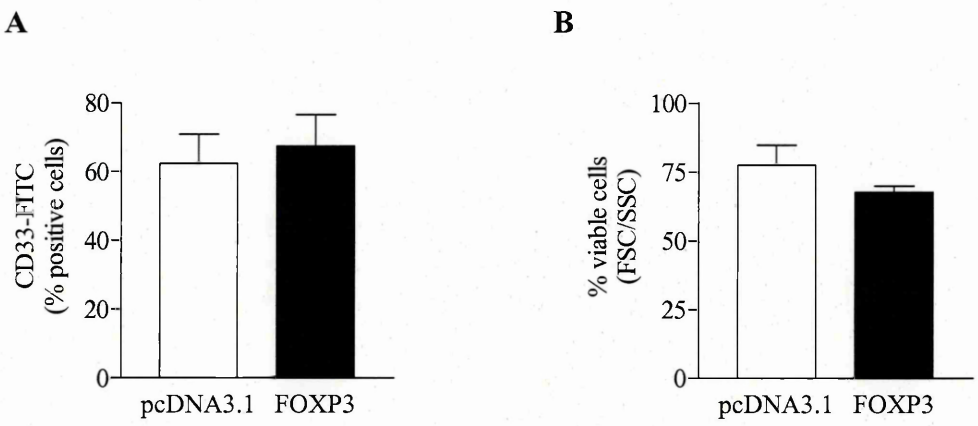


Figure 5.9 Measurement of CD4+ T cell viabilities and CD134/TCRζ chimeric receptor expression levels following co-transfection with *FOXP3* or control plasmid DNA.
Cells were transfected with CD134/TCRζ chimeric receptor DNA and either control plasmid DNA (pcDNA3.1) or *FOXP3* DNA and then rested for 4 hours prior to seeding onto 96-well plates. 24 hours post-transfection cells were analysed for CD134/TCRζ expression levels by using CD33-FITC (A). Cells were analysed for viability on the basis of forward and side scatter profiles (B). Results are mean ± SEM, n=3.

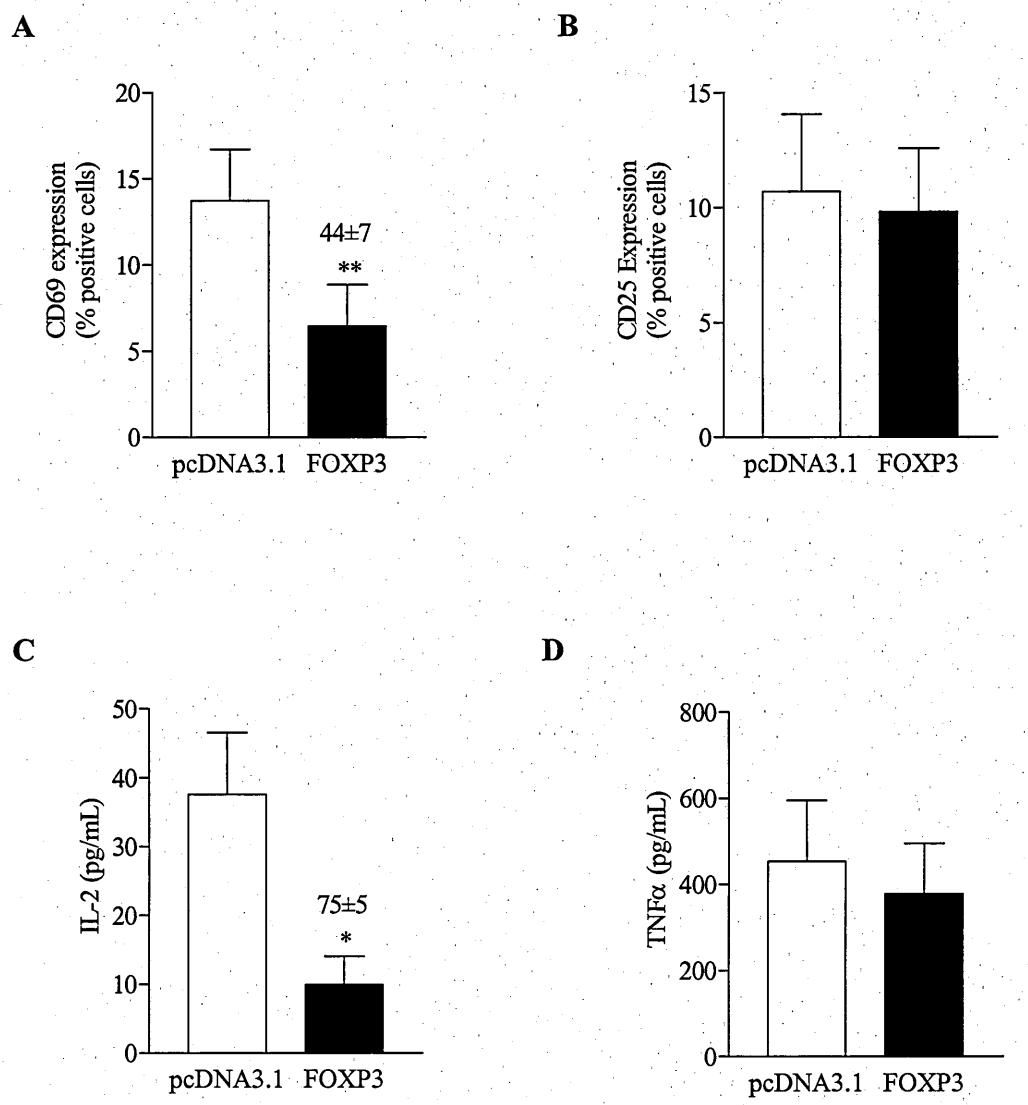


Figure 5.10 Measurement of CD4 T cell activation following co-transfection of CD134/TCRζ chimeric receptor DNA with either full-length *FOXP3* or control plasmid DNA.

Cells were transfected with CD134/TCRζ chimeric receptor DNA and either pcDNA3.1 or *FOXP3* DNA. Cells were rested for 4 hours and then seeded onto CD33-coated plates. Cells were removed 24 hours later and stained for CD69 expression levels (A) and CD25 expression levels (B). Supernatants were removed and assayed for IL-2 (C) and TNFα (D). Results are mean ± SEM, n=3. Values above the bars indicate percentage inhibition ± SEM. *, $p<0.05$; **, $p<0.001$ as compared with vector control (Paired T-test).

These studies have shown that overexpression of functional FOXP3 in human CD4+ T cells resulted in a significant inhibition of T cell activation, following stimulation through either the CD28/TCRζ or the CD134/TCRζ signalling pathways. CD28 is a member of the immunoglobulin-like CD28-B7 receptor family and is reported to signal through PI3-K (Alegre *et al.*, 2001). In contrast, CD134 is a member of the TNFR family and signals *via* the recruitment of various TNFR-associated factor (TRAF) signalling molecules (Arch *et al.*, 1998). ICOS like CD28 belongs to the B7 superfamily whilst CD137 (4-1BB) is an additional member of the TNFR family.

It was decided to investigate whether FOXP3 could also inhibit CD4+ T cell activation induced by these two co-stimulatory molecules using the chimeric receptors, ICOS/TCRζ and CD137/TCRζ (figures 5.11A and 5.11B). Published studies have confirmed the signalling components of both of these receptor are functional and lead to enhanced levels of IL-2 and TNFα production compared with a chimeric receptor containing the TCRζ signalling region alone (Finney *et al.*, 2004).

Human CD4+ T cells were transfected with ICOS/TCRζ or CD137/TCRζ chimeric receptor DNA and *FOXP3* or pcDNA3.1 (control vector) DNA. The FKH mutant was not used for these studies as it was unavailable. A proportion of the cells were then incubated for 24 hours without any further stimulation and stained for cell surface expression of the chimeric receptor (figure 5.12A). Viabilities were determined by FSC/SSC profiles and were found to be equivalent between the FOXP3 transfected cells and the cells transfected with control plasmid, with levels of around 70% viability being achieved for both chimeric receptor constructs (data not shown).

The remaining cells were seeded onto CD33 coated plates for 24 hours. Culture supernatants were then removed and assayed for IL-2 (figure 5.12B)

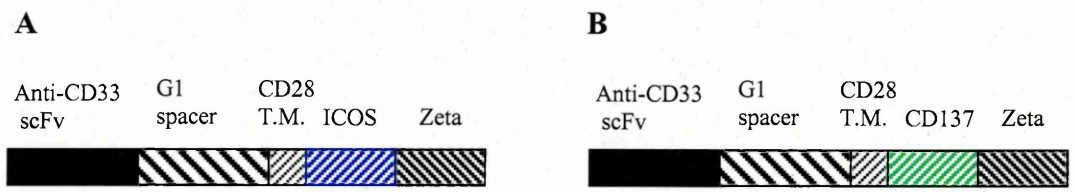


Figure 5.11 Schematic diagrams illustrating the ICOS/TCRζ and CD137/TCRζ chimeric receptors.
Both chimeric receptors consist of a single chain Fv specific for CD33, linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3. The spacer is linked to the transmembrane (T.M.) region of human CD28 and either the intracellular region of human ICOS (A) or CD137 (B), both of which are linked to the intracellular region of human TCRζ.

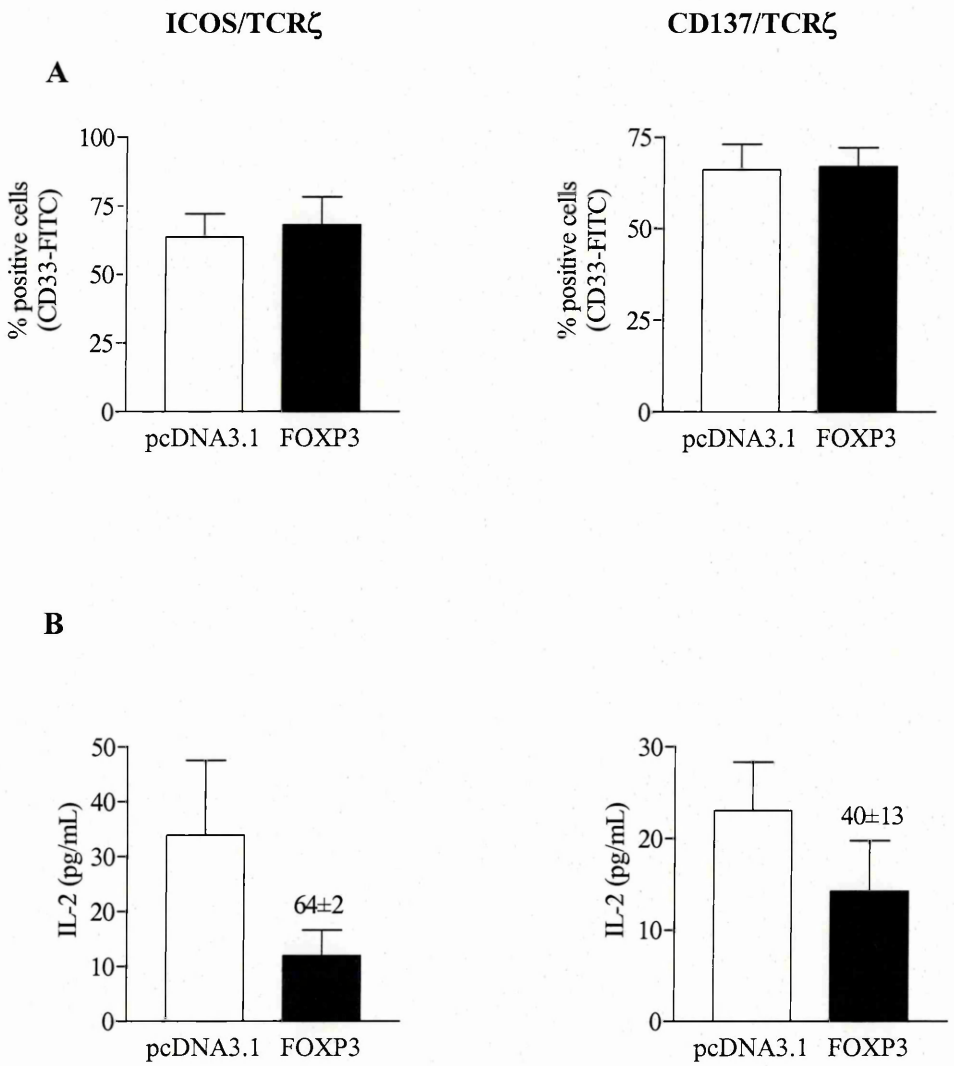


Figure 5.12 Measurement of chimeric receptor expression levels and CD4+ T cell activation following co-transfection of either ICOS/TCRζ or CD137/TCRζ chimeric receptors with either full-length *FOXP3* or control vector DNA.

Cells were transfected with ICOS/TCRζ or CD137/TCRζ chimeric receptor DNA and either pcDNA3.1 or *FOXP3* DNA. Half the transfected cells were incubated for 24 h at 37°C without CD33 stimulation and then stained for chimeric receptor expression levels with CD33-FITC (A). The remaining cells were rested for 4 hours and then seeded onto CD33-coated plates as before. Supernatants were removed 24 hours later and assayed for IL-2 (B). Results are mean ± SEM, n=3. Values above bars represent percentage inhibition ± SEM.

The data illustrated in figure 5.12 shows that both chimeric receptors were expressed at high levels in the purified human CD4+ T cells with expression levels of around 70% being achieved, 24 hours post-transfection. Levels of IL-2 production were much lower than those achieved with the CD28/TCRζ chimeric receptor. This was also observed following co-transfection of the CD134/TCRζ chimeric receptor. However this is not surprising as signalling through CD28 delivers a potent primary activation signal, whilst the other molecules are traditionally involved in later T cell activation events. Whilst not statistically significant, overexpression of FOXP3 did produce a trend towards inhibition of IL-2 production from both the ICOS/TCRζ and the CD137/TCRζ chimeric receptors. Experiments with more donors would have probably resulted in statistical significance being achieved.

5.2.5 Investigation into whether overexpression of FOXP3 in human CD4+ T cells leads to the generation of suppressive activity.

These data confirmed a potential role for FOXP3 as an inhibitor of T cell activation in both human CD4+ and CD8+ T cells. However recent data has shown that FOXP3 also appears to play a pivotal role in the generation of suppressive activity (Hori *et al.*, 2003; Khattri *et al.*, 2003; Fontenot *et al.*, 2003). Experiments were therefore carried out to determine whether this was also true of the cells being used in these studies. The literature suggests that for regulatory T cell function to manifest itself, there is a requirement both for FOXP3 expression and specific activation through the TCR (Zahorsky-Reeves and Wilkinson, 2002). In order to achieve this, the 'suppressor' cells had to be transfected with both FOXP3 and chimeric receptor. However the

responder cells, i.e. the cells to be suppressed, only required transfection with the chimeric receptor. Co-culture experiments were carried out using CD4+ T cells transfected with both FOXP3 and CD28/TCRζ, mixed in a 2:1 ratio with CD4+ T cells transfected with only CD28/TCRζ. Cells were seeded onto CD33-coated plates and 24 hours later, culture supernatants were removed and assayed for IL-2. The experimental protocol is illustrated in figure 5.13.

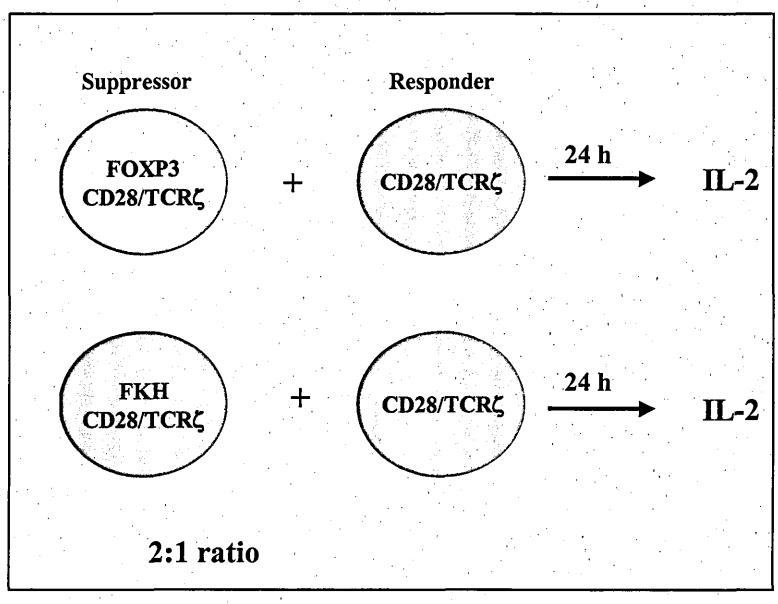


Figure 5.13 Schematic diagram of the experimental protocol followed for the FOXP3-CD28/TCRζ suppressor assay.
Purified human CD4+ T cells were co-transfected with CD28/TCRζ chimeric receptor DNA and either plasmid DNA encoding full-length *FOXP3* or the FKH mutant. The co-transfected cells were then mixed in a 2:1 ratio with cells transfected with the CD28/TCRζ chimeric receptor alone. The cells were seeded onto CD33-coated plates and incubated for 24 hours at 37°C. Supernatants were removed and assayed for IL-2.

Results from the FOXP3-CD28/TCRζ suppressor assay are illustrated in figure 5.14. Evidently in this system, the FOXP3 co-transfected cells were unable to suppress the activation of the CD28/TCRζ transfected cells. This led to other assay formats being explored. The first involved pre-activation of the FOXP3 co-transfected cells *via* the chimeric receptor for 24 hours, prior to co-culturing for a further 24 hours with freshly

transfected CD28/TCRζ CD4+ T cells on CD33-coated plates. The rationale for this strategy was that the activation of the signalling pathways required to generate suppressive activity mediated by FOXP3, might require longer than 24 hours to become fully functional. However no suppression of IL-2 production was observed (data not shown). This could be explained by the fact that these studies have previously shown that FOXP3 expression is very transient and decreases after 24 hours (data not shown).

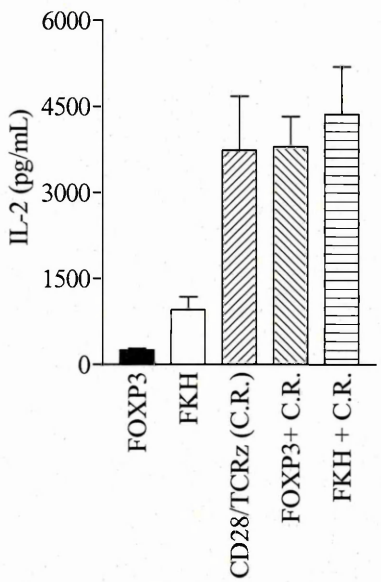


Figure 5.14 Measuring CD4+ T cell activation following the co-culture of cells co-transfected cells with *FOXP3* or FKH mutant DNA and CD28/TCRζ chimeric receptor, with cells transfected with CD28/TCRζ chimeric receptor alone.
Purified human CD4+ T cells were co-transfected with CD28/TCRζ chimeric receptor DNA and either plasmid DNA containing full-length *FOXP3* or the FKH mutant (designated FOXP3 or FKH). The co-transfected cells were then mixed in a 2:1 ratio with cells transfected with the CD28/TCRζ chimeric receptor alone (C.R.). The cells were seeded onto CD33-coated plates and incubated for 24 hours at 37°C. Supernatants were removed and assayed for IL-2. Results are mean ± SEM, n=3.

There was a concern that the levels of FOXP3 being expressed within the cell were not sufficient to inhibit both T cell activation within the co-transfected cells and to play a role in the additional cellular events required to initiate suppression of

neighbouring cells, if indeed these pathways are distinct signalling events. A new chimeric receptor that only has the intracellular signalling region of TCRζ was used in an attempt to address this issue (figure 5.15). It was believed that signalling through this receptor should be sufficient to reveal effects of FOXP3, but the overall level of activation within the cell would be low, therefore the majority of the FOXP3 protein would potentially be available to participate in other signalling events. However as is shown in figure 5.16, adoption of this strategy did not lead to the generation of FOXP3 specific suppressive activity. Interestingly, the co-culture conditions appeared to result in a non-FOXP3-dependent inhibition of chimeric receptor activation.

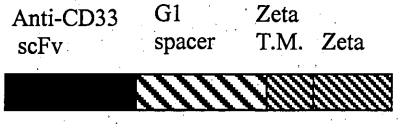


Figure 5.15 Schematic diagram illustrating the TCRζ chimeric receptor.

The chimeric receptor consists of a single chain Fv specific for CD33, linked to an extracellular spacer comprising human IgG1 hinge, CH2 and CH3. The spacer is linked to the transmembrane (T.M.) region of human TCRζ and the intracellular region of human TCRζ.

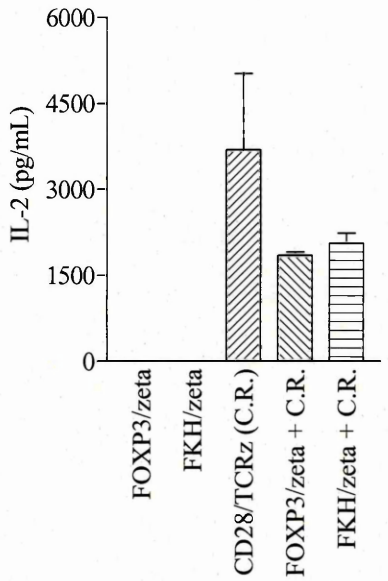


Figure 5.16 Measuring CD4+ T cell activation following the co-culture of cells co-transfected with *FOXP3* or FKH mutant DNA and TCRζ chimeric receptor, with cells transfected with CD28/TCRζ chimeric receptor alone.
Purified human CD4+ T cells were co-transfected with TCRζ chimeric receptor DNA and either plasmid DNA containing full-length *FOXP3* or the FKH mutant (designated FOXP3/zeta or FKH/zeta). The co-transfected cells were then mixed in a 2:1 ratio with cells transfected with the CD28/TCRζ chimeric receptor alone (C.R.). The cells were seeded onto CD33-coated plates and incubated for 24 hours at 37°C. Supernatants were removed and assayed for IL-2. Data shown are mean of 2 donors. Error bars represent minimum and maximum values.

It was possible that the activation of CD4+ T cells through the chimeric receptor signalling pathway generated a population of cells that were resistant to suppression. In an attempt to determine whether this was true, experiments were conducted investigating whether naturally occurring CD4+CD25+ regulatory T cells were able to inhibit CD28/TCRζ transfected CD4+ T cells. As already mentioned, CD4+CD25+ regulatory cells require activation through the TCR to be functional suppressor T cells, this meant a pre-activation step was required prior to co-culture with the chimeric receptor transfected cells. Freshly isolated CD4+CD25+ regulatory T cells were co-cultured for 24 hours in the presence of irradiated autologous CD4-depleted

PBMCs and 1μg/mL PHA-L. The CD4+ T cells were repurified using a MACS CD4+ negative isolation kit and then co-cultured in a 1:1 ratio with CD28/TCRζ transfected CD4+ T cells on CD33-coated plates for a further 24 hours. Culture supernatants were removed and assayed for IL-2. Pre-activated CD4+CD25- T cells were used as a control.

The data illustrated in figure 5.17 shows that pre-activated CD4+CD25+ regulatory T cells were capable of inhibiting T cell activation induced through chimeric receptor signalling. Levels of IL-2 measured in this assay system were considerably lower than those measured in the FOXP3-CD28/TCRζ suppressor assay (figure 5.14). This is because ten-fold less cells were used in these experiments due to poor recoveries of activated CD4+CD25+ regulatory cells following the purification step. These data together with the data presented in figure 5.16 suggest that chimeric receptor transfected cells are responsive to suppression.

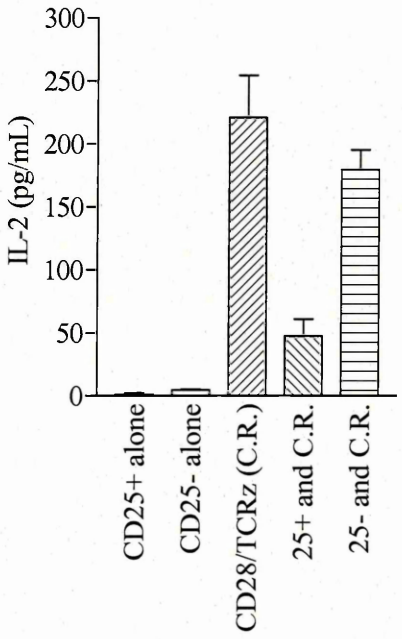


Figure 5.17 Measurement of IL-2 production following co-culture of CD28/TCRζ transfected CD4+ T cells with preactivated CD4+CD25+ regulatory T cells.
Purified human CD4+CD25+ and CD4+CD25- T cells were stimulated for 24 hours in the presence of irradiated autologous CD4-depleted PBMCs and 1μg/ml PHA-L. The cells were repurified using a MACS CD4+ negative selection isolation kit and mixed in a 1:1 ratio with CD4+ T cells transfected with CD28/TCRζ chimeric receptor DNA. The cells were seeded onto CD33-coated plates and incubated for 24 hours at 37°C. Supernatants were removed and assayed for IL-2. Data is representative of 3 independent experiments. Data is presented as mean cpm ± SD.

Another explanation for the lack of observed suppressive activity by the FOXP3-transfected cells, is that the CD28/TCRζ signalling pathway does not give the correct activation signals to reveal the suppressor function properties of FOXP3. This was unlikely as the chimeric receptor signalling pathway should mimic conventional T cell activation. However, to confirm that the chimeric receptor signalling pathway was sufficient to reveal FOXP3 suppressive activity, transfection experiments were undertaken using freshly isolated CD4+CD25+ regulatory cells. CD4+ T cells were isolated from peripheral blood as previously described and transfected with 3μg

CD28/TCRζ chimeric receptor DNA. Transfected cells were rested for 4 hours and then purified into CD25+ and CD25- fractions by using MACS CD25 microbeads. Transfected CD4+CD25+ cells were mixed in a 2:1 ratio with the transfected CD4+CD25- cells and seeded on CD33-coated plates. Cells were incubated for 24 hours and supernatants were removed and assayed for IL-2. A proportion of cells from each population were left unstimulated and viabilities and chimeric receptor expression levels were determined, 24 hours post-transfection.

The viability data displayed in figure 5.18A shows that the transfected CD4+CD25+ regulatory cells were significantly less viable than the transfected CD4+CD25- cells. Viable cells were then gated and the levels of chimeric receptor expression were measured. Figure 5.18B shows that the levels of CD28/TCRζ chimeric receptor expression were also slightly lower within the CD4+CD25+ regulatory T cell population. Nevertheless, the data shown in figures 5.19A and 5.19B reveals that the chimeric receptor-transfected CD4+CD25+ regulatory cells were capable of suppressing the transfected CD4+CD25- cells, resulting in a significant inhibition of activation-induced IL-2 production. These data confirm that signalling through the CD28/TCRζ chimeric receptor is sufficient to activate suppressive activity, potentially through the activation of endogenous FOXP3. Confirmation that the observed inhibition is not simply due to increased cell number following the co-culture of transfected CD25+ cells with CD25- cells, is shown in figure 5.19C where increasing numbers of chimeric receptor transfected CD25- were cultured on CD33-coated plates.

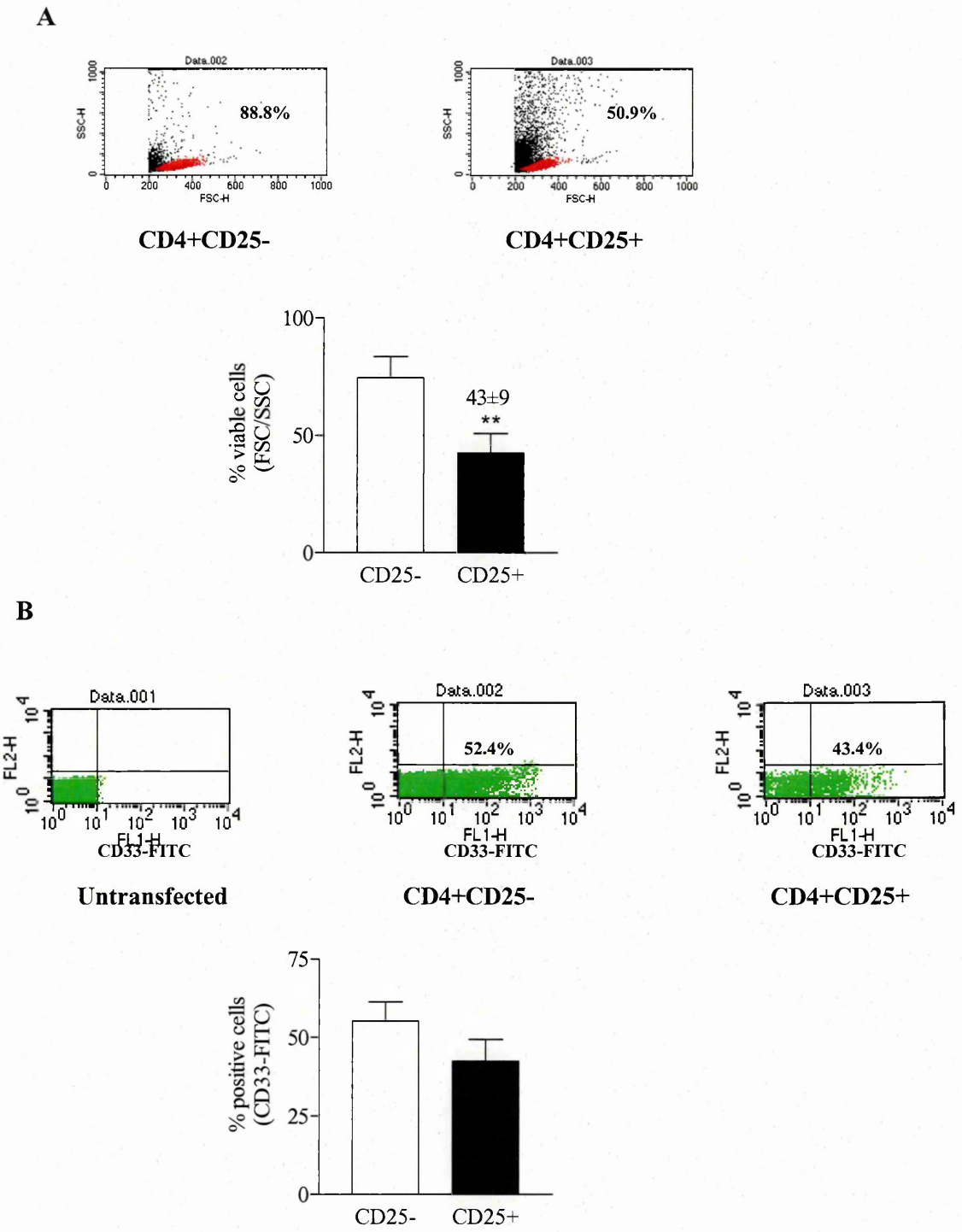


Figure 5.18 Measurement of cell viabilities and CD28/TCRζ chimeric receptor expression levels following transfection of CD4+CD25+ regulatory T cells and CD4+CD25- T cells.

Purified CD4+ T cells were transfected with 3μg of chimeric receptor DNA and rested for 4 hours. Cells were further purified into CD25+ and CD25- T cell populations using MACS CD25 microbeads and incubated for 24 hours at 37°C. Cell viabilities were determined using FSC/SSC profiles (A) and chimeric receptor expression was measured using CD33-FITC (B). Dot plots are representative of 1 of 5 donors analysed. Values on dot-plots indicate percentage viable cells (red) (A) and percentage positive cells expressing chimeric receptor (B) Bar graphs show combined data for 5 donors ± SEM. Values above the bars indicate percentage inhibition ± SEM. **, $p<0.001$ as compared with CD25- T cells (Paired T-test).

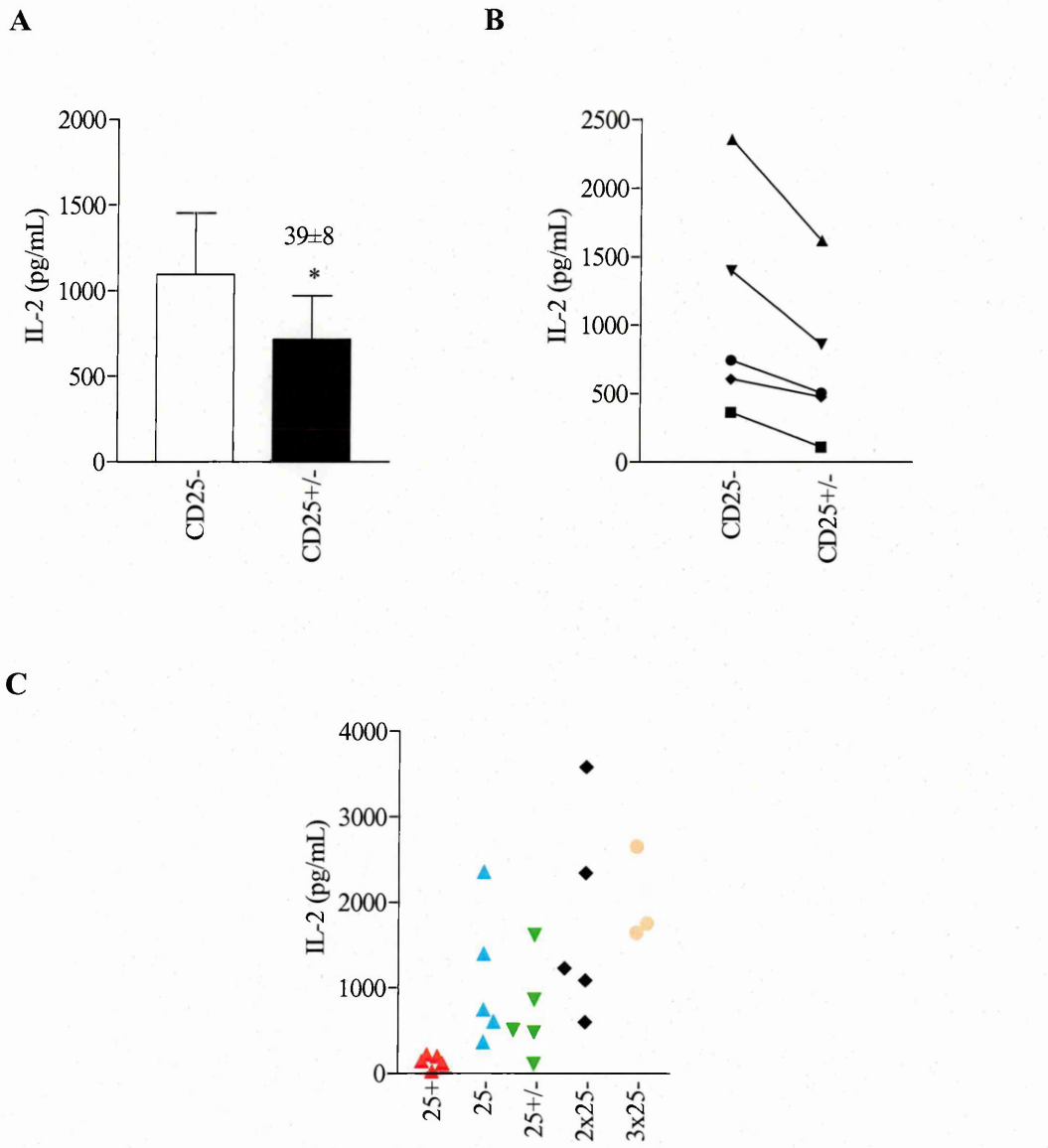


Figure 5.19 Measurement of CD4+ T cell activation following the co-culture of CD28/TCRζ transfected CD4+CD25+ regulatory T cells with CD28/TCRζ transfected CD4+CD25- T cells.

Purified CD4+ T cells were transfected with 3μg of chimeric receptor DNA and rested for 4 hours. Cells were further purified into CD25+ and CD25- T cell populations using MACS CD25 microbeads. The transfected CD25+ cells were then mixed in a 2:1 ratio with the transfected CD25- cells and seeded onto CD33-coated plates. 24 hours post-transfection, supernatants were removed and assayed for IL-2 (A and B). Increasing numbers of CD25- transfected cells were also seeded onto CD33-coated and supernatants were assayed for IL-2, 24 hours post-transfection (C). Results are mean ± SEM, n=5 with the exception of the data for 3xCD25- where data are n=3. Values above the bars indicate percentage inhibition ± SEM. *, $p<0.05$ as compared with transfected CD25- cells alone.

5.3 Discussion

Optimisation of the CD28/TCRζ chimeric receptor co-transfection system, described in the previous chapter, provided a basis for further investigation into the effect of overexpression of FOXP3 on subsequent human T cell activation. Evidence that FOXP3 plays a role in regulating T cell activation came initially from studies with CD4+ T cells isolated from the scurfy mouse. The CD4+ T cells were found to be hyperresponsive to TCR stimulation, resulting in the production of elevated levels of a number of cytokines including GM-CSF, IL-2, IL-4, IFNγ and TNFα (Clark *et al.*, 1999; Kanangat *et al.*, 1996). Conversely, characterisation of CD4+ T cells isolated from mice transgenic for the *Foxp3* gene, showed reduced proliferative responses to various stimuli and low levels of IL-2 production (Khattri *et al.*, 2001). Studies with the Jurkat T cell line had also shown that transient expression of FOXP3 resulted in an attenuation of activation-induced IL-2 production (Schubert *et al.*, 2001).

In an attempt to determine if FOXP3 played a role in human T cell activation, experiments were initially performed using freshly isolated peripheral blood-derived human CD4+ T cells. The CD4+ T cells were co-transfected with DNA encoding the CD28/TCRζ chimeric receptor and FOXP3. Stimulation of the transfected cells for 24 hours through the chimeric receptor, resulted in a significant inhibition of CD4+ T cell activation as measured by the release of both IL-2 and TNFα, and the levels of cell surface CD69 expression. These data confirmed a role for FOXP3 in regulating human CD4+ T cell activation and also confirmed that the FKH mutant was unable to suppress CD4+ T cell activation. This is believed to be because the FKH mutant is unable to translocate to the nucleus (Schubert *et al.*, 2001) and indeed the data

generated from the microscopy studies described in these studies supports this. Certainly the pattern of staining obtained indicated that the FKH mutant and the full-length FOXP3 protein were localised within different regions of the cell.

Interestingly, overexpression of FOXP3 did not affect the levels of CD25 cell surface expression. However, the levels of CD69 were inhibited and so it would not have been surprising for the CD25 levels to also be inhibited. The most likely explanation for this difference is that measuring CD25 expression 24 hours post-activation, was too early a time-point to see any difference in expression levels. CD25 expression typically peaks around 3 days after activation unlike CD69, which is an early activation marker. Alternatively there is data suggesting that CD25 expression is linked to FOXP3 expression. Retroviral transduction of FOXP3 into both mouse and human CD4+CD25- resulted in elevated levels of CD25 expression compared to control transduced cells (Hori *et al.*, 2003; Yagi *et al.*, 2004). Again it is possible that 24 hours post-activation may have been too early a time-point to observe this effect in these studies. However it is also important to note that retrovirally-transduced cells are pre-activated, therefore it is conceivable that FOXP3 does not induce CD25 expression but rather maintains its expression.

Although adoptive transfer experiments have shown that the disease in scurfy mice is mediated by CD4+ T cells (Blair *et al.*, 1994), a role for FOXP3 in CD8+ T cell function has been proposed by data generated from studies with CD8+ T cells isolated from mice transgenic for the *Foxp3* gene. These studies have shown that the transgenic CD8+ T cells have reduced cytolytic activity and are also defective in their ability to produce IL-2 (Khattri *et al.*, 2001). There is also evidence from the

literature, that sub-populations of both naturally occurring human and rat CD8+ T cells express FOXP3 (Walker *et al.*, 2003a; Xystrakis *et al.*, 2004). Indeed immunoblot data presented in this thesis supports the observation that a population of naturally occurring CD8+ T cells express FOXP3. This led to further studies being undertaken using CD8+ T cells instead of CD4+ T cells. Co-transfection of purified human CD8+ T cells with CD28/TCRζ chimeric receptor and FOXP3 resulted in a significant inhibition of chimeric receptor-induced T cell activation. These data support the concept that FOXP3 is a key regulator of T cell activation. Interestingly overexpression of FOXP3 in mouse B cells did not appear to affect B cell function (Khattri *et al.*, 2001). Polyclonal activation of B cells isolated from Foxp3 transgenic mice, revealed normal antibody responses. The transgenic B cells were also competent at generating antibody responses following immunisation with KLH, providing non-transgenic CD4+ T cells were present. These data suggest that either the signalling pathways required to activate FOXP3 are lacking in B cells or the downstream targets of FOXP3 are absent.

Optimal activation of lymphocytes requires an antigen specific signal (signal one) and a co-stimulatory signal (signal two). This two-signal model for lymphocyte activation was first proposed in 1970 (Bretscher and Cohn, 1970). Signal one is delivered following engagement of the peptide-MHC complex with the TCR. Signal two is delivered to T cells by co-stimulatory molecules expressed on the surface of APCs. Co-stimulation through CD28 appears to be crucial for the activation of naïve T cells. This activation results in high levels of IL-2 production, expansion of T cell numbers and resistance to apoptosis through the upregulation of BCL-X_L (Riley and June, 2005; Alegre *et al.*, 2001). Activation of memory T cells and effector T cells is

believed to be less dependent on CD28 signalling. Rather other molecules such as ICOS, CD134 (OX40) and CD137 (41-BB) are thought to play an active role in sustaining the immune response and in the generation of T-cell memory (Croft, 2003; Sharpe and Freeman, 2002). It was therefore decided to investigate whether FOXP3 was also capable of inhibiting CD4+ T cell activation initiated through these three alternative signalling pathways.

The data obtained using chimeric receptors specific for CD134, ICOS and CD137, confirmed that FOXP3 was capable of inhibiting CD4+ T cell activation initiated by all three of these alternative immunoregulatory pathways. Validation of the functionality of each of these receptors had been confirmed by studies showing enhanced antigen-induced IL-2 and TNFα production, compared with the chimeric receptor with TCRζ signalling alone (Finney *et al.*, 2004). It was observed both in these published studies and the studies presented in this thesis, that the levels of IL-2 produced following activation were much lower than those obtained following activation *via* the CD28 signalling pathway. However this is a reported phenomenon, with other cytokines being preferentially upregulated by these signalling pathways (Kroczek *et al.*, 2004; Sharpe and Freeman, 2002). These studies have shown that providing CD4+ T cells receive a signal through the TCR signalling pathway, FOXP3 can act as an inhibitor of T cell activation induced by a number of different co-stimulatory pathways. One minor caveat with measuring inhibition of IL-2 production as a measure of suppression is that inhibition could be due to IL-2 consumption. To resolve this issue, IFNγ could be measured or a blocking antibody to CD25 could be included in the assay.

The observation that FOXP3 inhibits T cell activation suggests that during the course of an immune response, one function of FOXP3 might be to limit the extent of the response following activation-induced upregulation. Evidence that FOXP3 is upregulated following CD4+ T cell activation is provided by studies which showed that culturing of CD4+CD25- T cells with plate-bound anti-CD3 and soluble anti-CD28 resulted in the upregulation of FOXP3 expression (Walker *et al.*, 2003a). Further evidence for a role for FOXP3 as a regulator in the periphery is supported by the phenotype of the *Foxp3* transgenic mouse. When *Foxp3* expression was under the control of the *lck* proximal promoter as opposed to its natural promoter, and thus only expressed during thymic development, the transgenic mice succumbed to the scurfy phenotype (Khattri *et al.*, 2001). These studies suggest the absolute requirement for continued expression of FOXP3 in the periphery to prevent lymphoproliferative disease.

Whilst these studies have shown that FOXP3 can act to directly inhibit T cell activation, it is now becoming apparent that FOXP3 plays a more complex role in controlling T cell activation and is linked to the biological activity of CD4+CD25+ regulatory T cells. Evidence for this link arose from a detailed analysis of the T cell populations isolated from both the scurfy mouse and mice transgenic for the *Foxp3* gene. Scurfy mice were found to lack CD4+CD25+ regulatory cells whereas the transgenic mice possessed an increased percentage of CD4+CD25+ regulatory cells (Khattri *et al.*, 2003). It was also interesting to observe that both the CD4+CD25- and CD4-CD8+ cells isolated from the transgenic mice had suppressive activity but not the B cells, again highlighting the inability of FOXP3 to generate regulatory activity in B cells.

Retroviral transduction of *Foxp3* into mouse CD4+CD25- T cells confirmed that overexpression of FOXP3 protein resulted in the generation of cells, which had regulatory activity both *in vitro* and *in vivo* (Hori *et al.*, 2003; Fontenot *et al.*, 2003). This has been further supported by recent data showing that a single injection of islet antigen-specific T cells transduced with *Foxp3,* was able to reverse disease in mice with recent-onset diabetes (Jaeckel *et al.*, 2005). All of the studies described thus far have investigated the effect of overexpression of FOXP3 in mouse T cells. It was therefore decided to investigate whether overexpression of FOXP3 in human CD4+ T cells also resulted in the generation of regulatory T cells.

Although overexpression of human FOXP3 resulted in a significant inhibition of both CD4+ and CD8+ T cell activation, these studies showed that the transient expression of FOXP3 was not sufficient to generate CD4+ T cells with suppressive properties. It is tempting to speculate that this is the result of an intrinsic difference between mouse and human CD4+ T cells. Certainly differences between mouse and human CD4+CD25+ regulatory T cells have been shown, including differences in cell surface marker expression and responsiveness to different cytokines, as highlighted earlier in these studies. One possibility is that overexpression of FOXP3 is necessary but not sufficient to convert a human CD4+ T cell into a regulatory T cell. However a recent publication has shown that retroviral transduction of human CD4+CD25- cells with FOXP3 did result in conversion of the cells into a regulatory phenotype (Yagi *et al.*, 2004). This suggests that the system used in the studies described in this thesis, may not be optimised sufficiently for the detection of regulatory function.

Additional experiments carried out within these studies confirmed that the activation of chimeric receptor transfected cells could be inhibited following co-incubation with pre-activated CD4+CD25+ regulatory T cells. Studies using CD4+ T cells transfected with TCRζ chimeric receptor and either FOXP3 or FKH mutant also appeared to partially inhibit the activation of CD4+ T cells transfected with CD28/TCRζ chimeric receptor. One possible explanation for this inhibition is that following the delivery of an activation signal through the TCRζ chain in the absence of a costimulatory signal, an anergic phenotype was induced within these cells. These cells were then able to partially suppress the response of the CD28/TCRζ transfected cells. Studies by Lombardi *et al.* have confirmed that some anergic human T cell clones are able to act as functional suppressor cells *in vitro* (Lombardi *et al.*, 1994).

Signalling through the chimeric receptor was also sufficient to deliver the necessary signals required for activation of regulatory function within naturally occurring CD4+CD25+ regulatory cells. Although not proven, this is likely to be due to activation of FOXP3 because FOXP3 is required for regulatory function. Together these data suggested that the lack of regulatory activity might have been due to the levels of FOXP3 expression that were achieved, or the interplay of the activation pathways used and the levels of FOXP3 transfected. Studies comparing the functional activity of CD4+CD25+ regulatory T cells isolated from *Foxp3* transgenic mice with CD4+CD25+ regulatory T cells isolated from wild-type mice support this theory. The transgenic cells were shown to express 10-15 times more mRNA for *Foxp3* than the wild-type cells, although the functional activity of the transgenic cells on an activity per cell basis was slightly reduced (Khattri *et al.*, 2003).

This is further supported by the observation that all the experiments in which overexpression of FOXP3 has been shown to generate regulatory function, have used expression systems that allow the continuous expression of FOXP3 protein (Hori *et al.*, 2003; Khattri *et al.*, 2003; Fontenot *et al.*, 2003; Yagi *et al.*, 2004). In some instances, this has also included an enrichment step to ensure only the highest producing cells were used (Yagi *et al.*, 2004; Hori *et al.*, 2003). In contrast, the system used in these studies only supports a short transient expression of FOXP3, which although sufficient to inhibit T cell activation, was perhaps not sufficient to reach the threshold required to also generate regulatory function.

Another key difference between the retroviral system and the nucleofection technique adopted in these studies is the activation state of the cells prior to transfection. These studies have used freshly isolated resting cells whereas for the retroviral studies, the cells required pre-activation. It is conceivable that certain pathways necessary to activate the regulatory function of FOXP3 were lacking in the resting cells or at the very least, required more than 24 hours stimulation to become fully functional. Attempts were made to co-transfect pre-activated CD4+ T cells with chimeric receptor and FOXP3, however the results obtained were variable and were not pursued. Recent data has been published showing that FOXP3-transfected Jurkats have suppressive activity (Choi *et al.*, 2005). However it should be also noted that Jurkats are in a permanent state of activation and therefore cannot be classed as resting T cells. Also the data was obtained using a stable selected Jurkat clone, so the levels of FOXP3 expression were constant.

In conclusion, the most likely explanation for the lack of suppressive activity observed in these studies, is probably due to the levels of FOXP3 expression that were achieved. The system described involved a very transient expression of the protein that peaked at 24 hours. There was also no enrichment or selection step for the very highest producing cells. To fully determine whether FOXP3 overexpression in resting human T cells does result in the generation of suppressive activity, studies need to be undertaken with viral vectors that are capable of infecting non-dividing cells, such as lentiviral vectors. This would ensure stable expression of the FOXP3 protein.

An important question that remains unanswered is how the overexpression of FOXP3 can switch a cell into becoming a CD4+CD25+ regulatory cell. There is evidence showing that activation through the TCR is required for FOXP3 to be functional, suggesting that either components of the TCR signalling cascade are responsible for regulating the activation of this protein, or FOXP3 acts on constituents of this pathway. However, this is not thought to involve direct phosphorylation of the FOXP3 protein because there is no consensus PKB/AKT site (Ramsdell, 2003). The activated FOXP3 protein may act as a transcriptional repressor, possibly by binding directly to FKH binding sites in the promoter regions of various pro-inflammatory genes and switching them off (Schubert *et al.*, 2001). It is feasible that this inhibition of activation pathways *via* the direct suppression of certain genes, then allows the expression of other as yet unidentified genes that are responsible for the regulatory phenotype. This theory suggests a sequential pathway of events. Recent data has suggested that FOXP3 induces the expression of heme oxygenase-1 (HO-1) and blockade of HO-1 using SiRNA to HO-1, was able to abrogate the suppressive function of a FOXP3 stably-transfected Jurkat T cell clone (Choi *et al.*, 2005).

Alternatively, activation through the TCR triggers two independent signalling pathways for FOXP3, one of which is involved in T cell activation and the other in T cell regulation. Clearly there are still many questions to be answered regarding the mechanism of action of FOXP3. The following chapter uses the chimeric receptor co-transfection system to investigate the functionality of two splice variant forms of the human FOXP3 gene that were identified during the cloning of the full-length gene.

Chapter 6

An Investigation into the Functionality of Splice Variant Forms of the Human *FOXP3* Gene

6.1 Introduction

The first forkhead (FKH) transcription factor was discovered in the fruit fly *Drosophila melanogaster* over ten years ago (Weigel *et al.*, 1989). These proteins are characterised by the presence of a 'winged helix' DNA binding domain, similar to the shape of a butterfly, which constitutes the FKH domain (Clark *et al.*, 1993). Over 100 different proteins with FKH domains have been identified and these have been subdivided into 17 different classes on the basis of the amino acid sequence of the FKH domain (Lehmann *et al.*, 2003). One characteristic of these proteins is that they can all bind DNA, although the functional effect of this can either be the transactivation or inhibition of gene transcription (Coffer and Burgering, 2004).

FOXP3 is a member of the forkhead-winged helix family of transcription factors and is believed to act as a transcriptional repressor. Indeed experimental data generated in these studies have confirmed the ability of FOXP3 to inhibit T cell activation. These studies have also confirmed the absolute requirement for the functional C-terminal forkhead domain for inhibition to occur. Other important structural domains present within the FOXP3 protein include a proline-rich region, a zinc finger domain and a leucine-zipper motif (Gambineri *et al.*, 2003).

Proline-rich motifs are believed to be important in protein-protein interactions. They tend to be exposed on the outer face of the protein thus allowing fast on and off-rates for binding (Kay *et al.*, 2000). Proline-rich regions are often found in situations where there is a requirement for rapid recruitment or interchange of several proteins, as occurs during the initiation of transcription and in signalling cascades. These regions

are also believed to participate in 'adaptor' systems where they can act as an adaptor to bring together other proteins (Kay *et al.*, 2000). Zinc finger domains have been reported to have many different functions including interactions with DNA, RNA and protein. To stabilise the structure of the zinc finger, these domains require the presence of one or more zinc ions (Laity *et al.*, 2001). A leucine-zipper typically consists of a stretch of 35 amino acids containing 4-5 leucine residues separated from each other by 6 amino acids (Landschulz *et al.*, 1988). This protein domain promotes dimer formation and these dimers can either be homodimers or heterodimers (Busch and Sassone-Corsi, 1990). The formation of dimers can be important for efficient DNA and protein binding (Abel and Maniatis, 1989).

During the cloning of the full-length version of the human *FOXP3* gene, as described in chapter 4, two novel splice variant forms of the gene were identified. Mapping studies described in the following results section, showed that the isoform referred to as clone 2, was found to lack exon 2, a region mapping within the proline-rich part of the gene. The other isoform, referred to as clone 10, lacked both exons 2 and 7. Exon 7 maps to a region forming part of a leucine-zipper structure. Since the discovery of these two splice variants, there have been two published reports showing expression of the isoform lacking exon 2 in human CD4+CD25+ regulatory T cells (Yagi *et al.*, 2004; Scotto *et al.*, 2004). However there is currently no published functional data using this variant. There is also no published data identifying the second isoform that lacks both exons 2 and 7. Thus the functionality of the splice variant forms of the human FOXP3 protein are currently unknown.

The aim of this chapter was to determine whether the two isoforms of FOXP3 were

functional inhibitors of CD4+ T cell activation. In order to determine this, cotransfection experiments were carried out using the previously described CD28/TCRζ chimeric receptor activation system. In addition, an investigation into whether splice variant forms of the *Foxp3* gene existed in mouse CD4+CD25+ regulatory T cells was subsequently undertaken.

6.2 Results

6.2.1 Mapping the missing regions within the FOXP3 splice variants

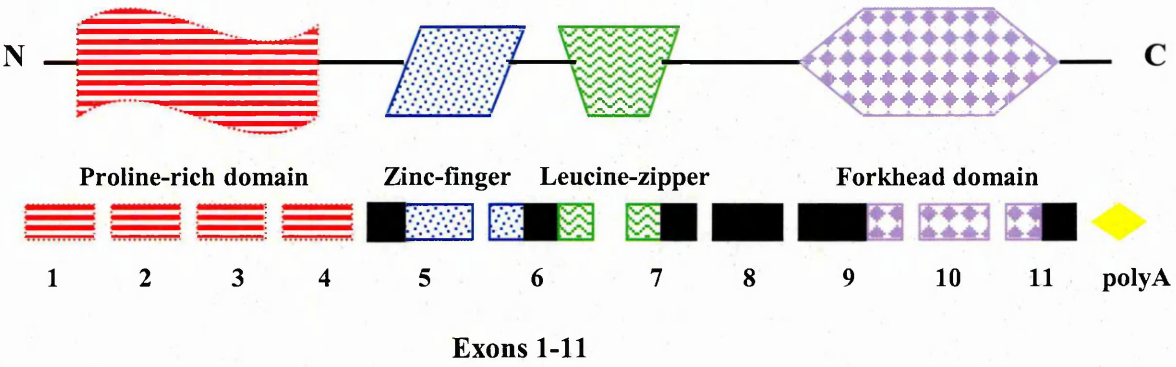
As discussed in chapter 4, amplification of full-length human *FOXP3* from human PBMC cDNA resulted in the generation of three different sized PCR products. These PCR products were cloned into the expression vector pTracer-CMV2 and sequenced. Sequence analysis revealed the presence of two splice variant forms of the gene as well as a full-length version (clone 4). Clone 2 was found to be lacking a region of 105 bases and clone 10 whilst also missing this 105 base region, lacked an additional region of 81 bases.

The human full-length *FOXP3* gene is 1296bp in size and has been reported to consist of 11 different exons (Gambineri *et al.*, 2003). The Ensembl Exonview mapping programme used to predict the exon structure of the FOXP3 protein, also suggested the existence of 11 coding exons. This exon predicition programme also enabled the missing nucleotide regions within the splice variants to be mapped to specific regions within the protein. The missing 105 bases (35 amino acids) identified in both clones 2 and 10 corresponded to exon 2 and the missing region of 81 bases (27 amino acids) identified in clone 10, corresponded to exon 7 (figure 6.1).

```
  1 MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGARGPGGTFQGRDLRGGAHASSSS
 61 LNPMPPSQLQLPTLPLVMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAHARTPVLQV
121 HPLESPAMISLTPPTTATGVFSLKARPGLPPGINVASLEWVSREPALLCTFPNPSAPRKD
181 STLSAVPQSSYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEKGRAQCLLQREMVQ
241 SLEQQLVLEKEKLSAMQAHLAGKMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE
301 APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMRPPFTYATLIRWAILEAPEKQRTL
361 NEIYHWFTRMFAFFRNHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEFRKKRSQR
421 PSRCSNPTPGP
```

Figure 6.1 Human FOXP3 Exon Structure
A predicted exon structure for the human FOXP3 protein sequence, calculated using the Ensembl Exonview mapping programme. The regions encoded by the 11 exons are depicted in alternating black and blue colours. Exons 2 and 7 are highlighted in bold. The amino acids depicted in red represent residue overlap splice sites, i.e. the new exon starts within the coding region of an amino acid.

A proposed structure of the human FOXP3 protein is shown in figure 6.2. The schematic diagram shows that in addition to the functional FKH domain at the C-terminus of the protein, the protein is also thought to consist of a proline-rich region, a zinc-finger and a leucine zipper. The exons believed to be responsible for coding the different regions are also shown. Based on this proposed structure, it appeared that clone 2 was lacking a proportion of the proline-rich domain, a region important in protein-protein interactions (Kay *et al.*, 2000). Clone 10 however, in addition to missing a large proportion of the proline-rich domain, also appeared to lack a large proportion of the leucine-zipper. The presence of a leucine-zipper indicates the possibility that FOXP3 forms dimers (Johnson and McKnight, 1989).



Adapted from IPEX, A Syndrome of Systemic Autoimmunity caused by mutations of *FOXP3*, a critical regulator of T cell homeostasis.
Gambineri *et al* 2003
Current Opinion in Rheumatology 15:430-435

Figure 6.2 Schematic diagram of the human FOXP3 protein.
Structure of the human FOXP3 protein schematically represented, highlighting the important structural domains and the corresponding exons encoding them.

6.2.2 Subcloning the FOXP3 splice variants

Before a detailed investigation into the functionality of the splice variants could occur, the genes had to be subcloned from the expression vector pTracer-CMV2 into the expression vector pcDNA3.1+. The rationale for the subcloning was because transfection studies described in chapter 4 had shown poor expression of the pTracer-CV2 plasmid in human CD4+ T cells, whereas the co-transfection system consisting of the pcDNA3.1 plasmid containing *FOXP3*.flag and the CD28/TCRζ chimeric receptor, had been successful. At this stage it was also decided to reclone the full-length *FOXP3* clone (clone 4) from pTracer-CMV2. The rationale for doing this was to confirm that the inhibition of T cell activation observed thus far, was due to the

functional effects of FOXP3 and was not influenced by the presence of the C-terminal flag tag.

Each of the three different variant forms of the *FOXP3* gene were excised from pTracer-CMV2 using the restriction enzymes *Eco*RI and *Not*I. The cloning vector, pcDNA3.1+ was also digested with these enzymes. Following a phenol-chloroform extraction, the digested fragments were separated by gel electrophoresis on 1% TAE agarose gels and the correct sized fragments were excised under low wavelength uv light. The gel fragments were purified using a Qiagen gel extraction kit and ligations were set-up overnight at 16°C. Ligated DNA, both vector alone and vector containing *FOXP3* DNA (insert) were transformed separately into chemically competent *E.coli*. As the transformation appeared to be successful, only six colonies were selected for culture from each *FOXP3* clone. Plasmid DNA was purified and diagnostic digests were carried out using the restriction enzymes *Eco*RI and *Not*I. The reactions were then separated by gel electrophoresis on 1% TAE agarose gels. All the plasmids appeared to contain inserts corresponding to the expected sized version of the *FOXP3* gene.

Large-scale DNA preparations were made of each of the 3 different *FOXP3* clones and a further *Eco*RI/*Not*I double digest was carried out to confirm the presence of the correct sized inserts. Figure 6.3 confirms that each *FOXP3* clone was successfully sub-cloned into the expression vector pcDNA3.1. Clone 4 generated an insert fragment of 1.296kb corresponding to the full-length gene, whereas clone 2 yielded a fragment of 1.191kb and clone 10 a fragment of 1.11kb. Sequence analysis further confirmed that the sequences were as expected (data not shown).

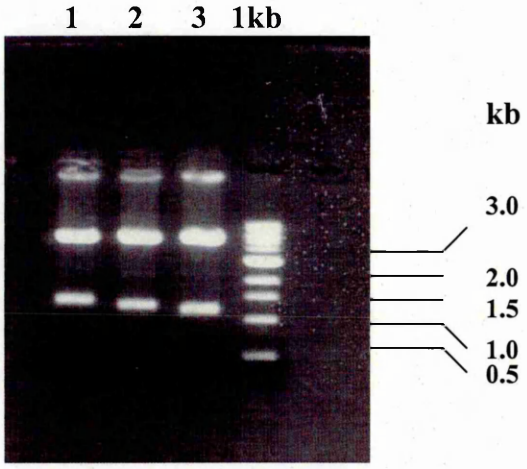


Figure 6.3 Diagnostic digests of plasmid DNA preparations containing either full-length *FOXP3*, clone 2 or clone 10.
Large scale DNA preparations of the full-length *FOXP3* clone (lane 1) and both clones 2 (lane 2) and 10 (lane 3) were prepared. Diagnostic digests were carried out using the restriction enzymes *Eco*RI and *Not*I. The digested fragments were separated on a 1% TAE agarose gel to confirm each plasmid preparation contained the expected sized variant form of the *FOXP3* gene.

6.2.3 Expression of the FOXP3 splice variants

Prior to studying the functionality of the splice variants, it was important to show that each variant was capable of being successfully translated. Initially this was confirmed using CHO cells. Transfections were carried out using 3μg of plasmid DNA encoding a variant of FOXP3, per $1x10^6$ cells. Cells were incubated at 37°C for 24 hours. Cell lysates were then prepared and assayed for total protein content. Equal amounts of total protein were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3. The immunoblot in figure 6.4 shows that both splice variant forms of the *FOXP3* gene were successfully translated.

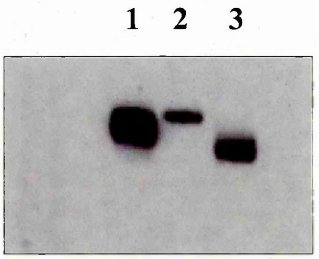


Figure 6.4 Expression of different variant forms of the FOXP3 protein in CHO cells. CHO cells were transfected with pcDNA3.1+ plasmid DNA containing either full-length *FOXP3* (lane 2), clone 2 (lane 1) or clone 10 (lane 3). 24 hours post-transfection, cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. Data shown is from one of two experiments which produced similar results.

It was also important to confirm that the expression levels of each FOXP3 variant were equivalent before any conclusions could be drawn with regard to functionality. Purified human CD4+ T cells were transfected with 9μg of plasmid DNA containing *FOXP3* variant DNA or FKH mutant DNA, per $4x10^6$ cells. Cells were incubated at 37°C for 24 hours. Cell lysates were then prepared and assayed for total protein content. Equal amounts of total protein were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3. The immunoblot in figure 6.5 shows that both splice variants are expressed in human CD4+ T cells. However the levels of expression of both clone 2 and clone 10 appeared to be slightly higher than both the full-length FOXP3 protein and the FKH mutant. This also appears to be true for the immunoblot shown in figure 6.4. One explanation for this may be that the absence of exon 2 renders the protein more stable and resistant to degradation, thus giving the appearance of increased expression levels. Nevertheless, it was felt that because the

full-length protein and the FKH mutant were expressed at comparable levels, it would still be valid to draw conclusions from the functional studies.

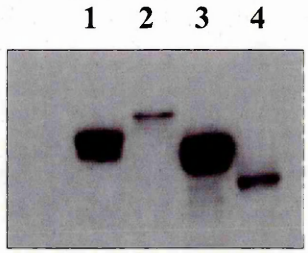


Figure 6.5 Expression of different variant forms of the FOXP3 protein in human CD4+ T cells.
Human CD4+ T cells were purified from peripheral blood using a MACS CD4+ negative selection isolation kit. The CD4+ T cells were transfected with pcDNA3.1+ plasmid DNA containing either full-length *FOXP3* (lane 2), clone 2 (lane 1), clone 10 (lane 3) or FKH mutant (lane 4). 24 hours post-transfection, cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. Data shown is from one of two experiments which produced similar results.

In chapter 4 immunoblot analysis of human CD4+CD25+ regulatory cells revealed that FOXP3 appeared to migrate as a doublet (figure 4.2). Following the identification of splice variant forms of the FOXP3 protein it was likely that the doublet represented both full-length FOXP3 and at least one of the splice variant forms. In an attempt to confirm this cell lysates were prepared from both human CD4+CD25+ and CD4+CD25- T cells and from CD4+ T cells transfected with either full-length *FOXP3*, clone 2 or clone 10. Cell lysates were then prepared and assayed for total protein content. Equal amounts of total protein were then loaded onto 10% (w/v) tris-glycine SDS PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3. Although the loading of the immunoblot in figure 6.6 does not appear to be equal, it shows that the upper band of the doublet present in freshly isolated CD4+CD25+ regulatory cells does appear to correspond to

full-length FOXP3. Conversely, the lower band of the doublet appears to correspond to clone 2. There is also a hint of a third band which may represent clone 10, however due to the loading of clone 10, this is difficult to confirm. From these data, it appears that a variant form of FOXP3 that lacks exon 2 does appear to be expressed in naturally occurring CD4+CD25+ regulatory cells.

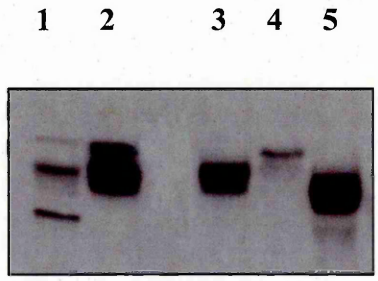


Figure 6.6 Expression of FOXP3 in freshly isolated human CD4+CD25+ and CD4+CD25- T cells and in CD4+ T cells transfected with different variant forms of *FOXP3* DNA.
Human CD4+ T cells were purified from peripheral blood using a MACS CD4+ negative selection isolation kit. A proportion of the CD4+ T cells were transfected with pcDNA3.1+ plasmid DNA containing either full-length *FOXP3* (lane 4), clone 2 (lane 3) or clone 10 (lane 5). 24 hours post-transfection, cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. The remaining CD4+ T cells were further purified into CD25+ (lane 2) and CD25- (lane 1) fractions using MACS CD25 microbeads and positive selection. Cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody. Data shown is from one of two experiments which produced similar results.

6.2.4 Determination of the functionality of the splice variants

The immunoblots in figure 6.4 and 6.5 confirmed that the splice variants were being expressed as protein. The next question to answer was whether this protein was functional. It was decided to use the chimeric receptor co-transfection system to investigate the functionality of the splice variants. As for the previous experiments using this system, it was important to confirm that both the viabilities of the transfected CD4+ T cells and the expression levels of the chimeric receptor were equivalent for each transfected cell population.

Purified CD4+ T cells were co-transfected with 3μg of CD28/TCRζ chimeric receptor DNA and 9μg of either full-length *FOXP3* DNA (clone 4), clone 2 DNA, clone 10 DNA or FKH mutant DNA. Viabilities were determined 24 hours post-transfection by the levels of PI uptake. Detection of the chimeric receptor was carried out using soluble CD33 labelled with Alexa Fluor 488. Figure 6.7A and 6.7B illustrate the data obtained following analysis by flow cytometry of transfected cells from one individual donor. Figure 6.7A shows the viability of the cells on the basis of PI uptake. The levels of chimeric receptor expression are shown in figure 6.7B. The combined results for seven different donors are illustrated in the figures 6.7C and 6.7D. Figure 6.7C shows that the viabilities of the four transfected populations were equivalent with viability levels of around 60-70% being achieved. The levels of CD28/TCRζ chimeric receptor expression were also equivalent between the different groups (figure 6.7D).

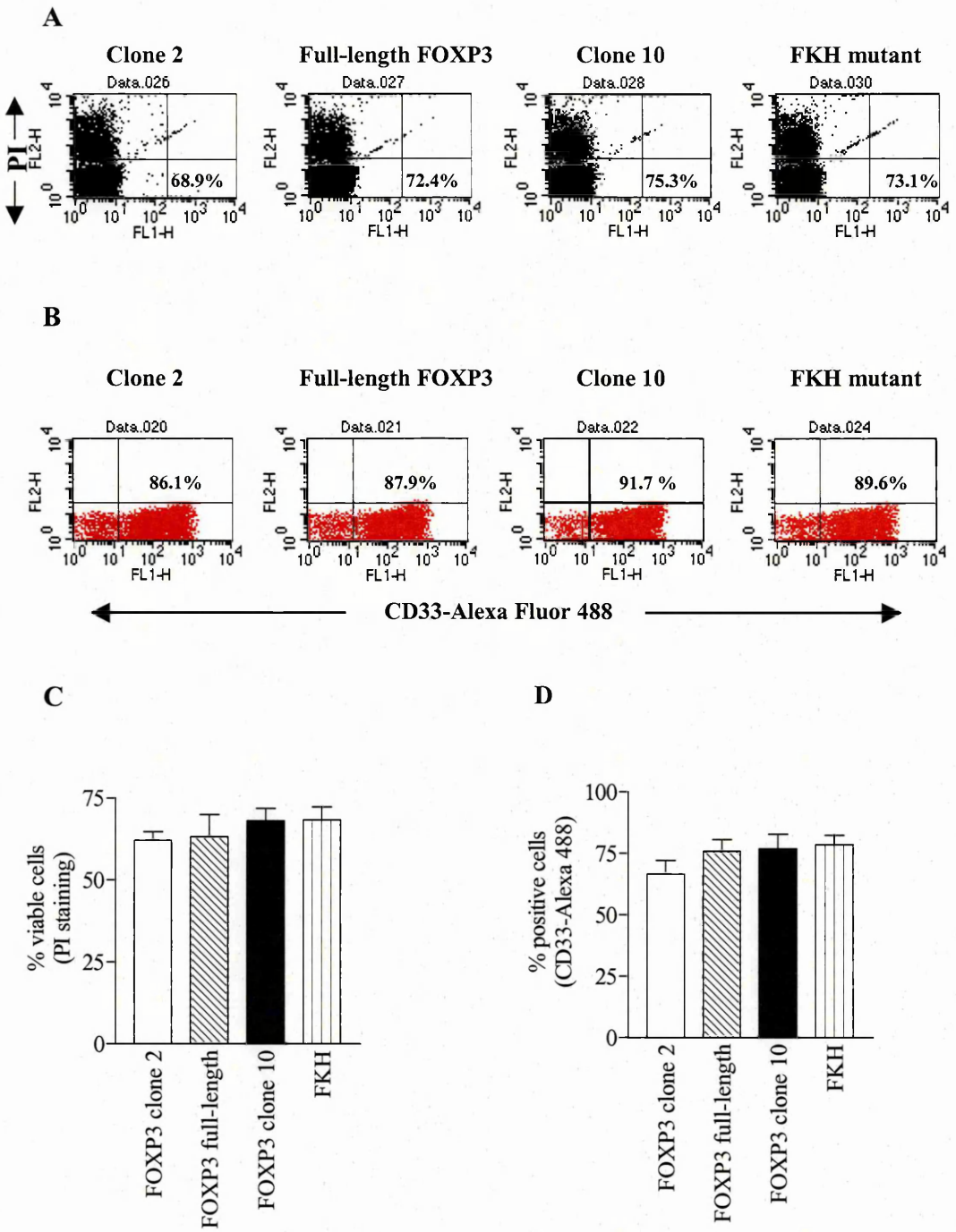


Figure 6.7 Measurement of CD4+ T cell viabilities and chimeric receptor expression levels following co-transfection with different variant forms of *FOXP3* DNA or FKH mutant DNA.

Human CD4+ T cells were co-transfected with CD28/TCRζ chimeric receptor DNA and either DNA encoding a variant form of *FOXP3* or FKH mutant. 24 hours post-transfection, cell viabilities were determined using PI staining (A). Values on dot-plots indicate percentage viable cells. Chimeric receptor expression was measured using CD33-Alexa Fluor 488 (B). Values on dot-plots indicate percentage positive cells expressing chimeric receptor. Combined data for cell viability are mean ± SEM, n=7 (C). Combined data for CD33 expression are mean ± SEM, n=7 (D).

Once it had been established that the transfection efficiency and the viabilities were broadly equivalent for the four different transfected cell populations, subsequent experiments concentrated on investigating whether the splice variants had any functional effect on signalling through the CD28/TCRζ chimeric receptor. Human CD4+ T cells were co-transfected with DNA encoding the CD28/TCRζ chimeric receptor and DNA encoding either a variant form of the *FOXP3* gene or FKH mutant and seeded onto CD33-coated plates. The culture supernatants were harvested 24 hours post-transfection and assayed for human IL-2. Cells were removed and stained for cell surface CD69 and CD25.

Figure 6.8A shows that there was a significant inhibition of IL-2 production by both FOXP3 variants and the full-length version of the protein, as compared to the FKH mutant. Figure 6.8B shows that both splice variant forms of the human FOXP3 protein were also able to inhibit the upregulation of CD69 expression following activation through the chimeric receptor. The levels of CD25 expression on the surface of cells expressing either a variant form of the FOXP3 protein or the FKH mutant were not significantly different (Figure 6.8C). Together these data suggest that the two splice variants forms of the human FOXP3 protein are functional inhibitors of CD4+ T cell activation and confirm that the absence of a C-terminal flag tag does not affect the ability of full-length FOXP3 to inhibit CD4+ T cell activation.

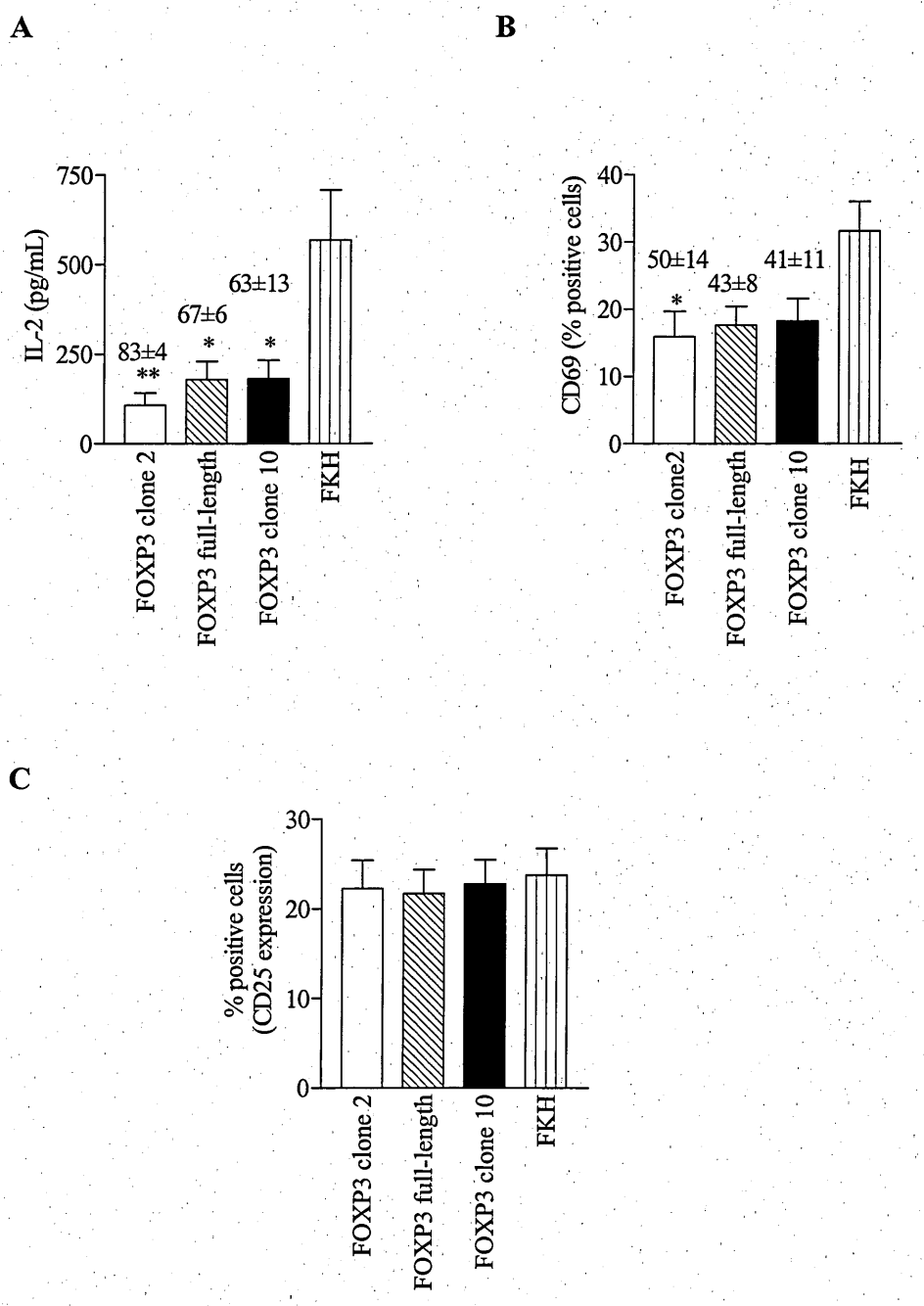


Figure 6.8 Measurement of CD4+ T cell activation following co-transfection of CD28/TCRζ chimeric receptor with different variant forms of human *FOXP3* DNA or FKH mutant DNA.

Human CD4+ T cells were co-transfected with CD28/TCRζ chimeric receptor DNA and either DNA encoding a variant form of *FOXP3* or FKH mutant DNA and then rested for 4 hours post-transfection. Cells were then seeded onto CD33-coated plates and incubated for 24 h at 37°C. Supernatants were removed and assayed for IL-2 (A). Cells were removed and stained for CD69 expression levels (B) and CD25 expression levels (C). Results are mean ± SEM, n=7 for CD69 and CD25 and n=8 for IL-2. Values above the bars represent percentage inhibition ± SEM. Statistical analysis was by Anova with Bonferroni post-test. **, $p<0.01$ versus FKH control, *,$p<0.05$ versus FKH control.

6.2.5 Investigation into whether splice variant forms of the Foxp3 gene occur in the mouse

The immunoblot of human CD4+CD25+ regulatory cells shown in figure 6.6 suggested that a variant form of human FOXP3 lacking exon 2 appears to be expressed naturally. This led to further studies to investigate whether variant forms of the protein are also expressed in mouse CD4+CD25+ regulatory cells. Mouse CD4+ T cells were purified from BALB/c splenocytes using a MACS CD4+ negative selection isolation kit. The CD4+ T cells were then further purified into CD25+ and CD25- fractions using the CD25 positive selection isolation kit. Cell lyates were prepared and assayed for total protein content. Equal amounts of protein were then loaded onto 10% (w/v) tris-glycine SDS-PAGE gels and separated by gel electrophoresis. Following transfer onto PVDF membrane, the blots were probed with a rabbit polyclonal antisera raised against recombinant human FOXP3 (cross-reacts with mouse FOXP3). The immunoblot illustrated in figure 6.9 shows that mouse FOXP3 protein appears to migrate predominantly as a single band suggesting only one form of the protein is highly expressed. This is in contrast to the human FOXP3 protein, which is clearly highly expressed as a doublet, possibly even multiple bands. Published data using rabbit polyclonal antisera raised against recombinant mouse FOXP3 confirms that the FOXP3 is expressed as a single band within mouse CD4+CD25+ regulatory T cells (Fontenot *et al.*, 2003). However, certainly in the studies described in this thesis, there is a very faint band corresponding to the lower band of the FOXP3 doublet in the human CD4+CD25- T cell population and both the CD25- and CD25+ populations of mouse CD4+ T cells. The physiological relevance of this is as yet unclear.

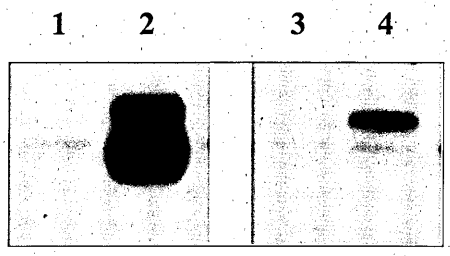


Figure 6.9 Expression of FOXP3 in human and mouse CD4+CD25+ and CD4+CD25- T cells.
Human CD4+ T cells were purified from peripheral blood using a MACS CD4+ negative selection isolation kit. The CD4+ T cells were further purified into CD25- (lane 1) and CD25+ (lane 2) fractions using MACS CD25 microbeads and positive selection. Mouse CD4+ T cells were purified from splenocytes and were further separated into CD25- (lane 3) and CD25+ (lane 4) fractions using a CD25 isolation kit. Cell lysates were prepared and analysed by Western blotting using rabbit polyclonal antisera to human FOXP3. Visualisation of the immunoreactive proteins was achieved using chemiluminescence after incubation of the blot with a goat anti-rabbit HRP-conjugated antibody.

A protein alignment of the mouse and human FOXP3 proteins is shown in figure 6.10. Overall there is 86.1% sequence identity between the two proteins. A sequence alignment of the mouse and human *Foxp3/FOXP3* genes is shown in figure 6.11. At the nucleotide level, there is 84.4% sequence identity. The high degree of sequence homology suggests a high degree of evolutionary conservation between the two species, signifying the importance of this protein in T cell regulation. In an attempt to determine whether variant forms of the mouse gene exist, it was decided to clone the mouse *Foxp3* gene into the expression vector pcDNA3.1+. The published sequence (Genbank accession number NM_054039) was used to design primers muFoxp3FWD (5' primer) and muFoxp3REV (3' primer) to allow amplification of the mouse *Foxp3* gene. Design of the 5' oligonucleotide included an *Eco*RI site to facilitate cloning and a partial Kozak translational initiation sequence for optimal gene expression, upstream of the initiator methionine. The 3' oligonucleotide contained a stop codon and a *Not*I restriction site. An in-house source of BALB/c mouse cDNA generated

from ConA activated T-cells was used to clone the gene. PCR conditions selected were the same as those described for cloning of the human *FOXP3* gene (section 4.2.2 Cloning of human *FOXP3*).

The large-scale PCR reaction was successful and the PCR product was subjected to a Qiagen Qiaquick PCR clean-up step to remove contaminants from the PCR reaction. Both the PCR product and the cloning vector (pcDNA3.1+) were then digested with *Eco*RI and *Not*I and following a second Qiagen Qiaquick clean-up step, the digests were separated on a 1% TAE agarose gel by gel electrophoresis and the bands excised under low wavelength uv light. The PCR fragment was excised as a large gel slice to ensure any variant forms of the gene were not excluded. The gel fragments were purified using a Qiagen gel extraction kit and ligations were set-up overnight at 16°C.

The ligated DNA, both vector alone and vector containing mouse *Foxp3* DNA (insert) were transformed separately into chemically competent *E.coli*. The transformation appeared to be successful, therefore forty-four individual colonies were cultured and plasmid DNA was purified. To identify which clones contained the full-length *Foxp3* gene, diagnostic digests were carried out using the restriction enzymes, *Eco*RI and *Not*I. The reactions were then separated by gel electrophoresis on a 1% TAE gel. Figure 6.12 shows a representative gel of 12 individual clones following restriction enzyme digestion. Each of the forty-four clones yielded an identical sized fragment of approximately 1.3kb. This suggested that if variant forms of the mouse *Foxp3* gene were present, they were present only at a very low frequency in the source of cDNA used to amplify the gene.

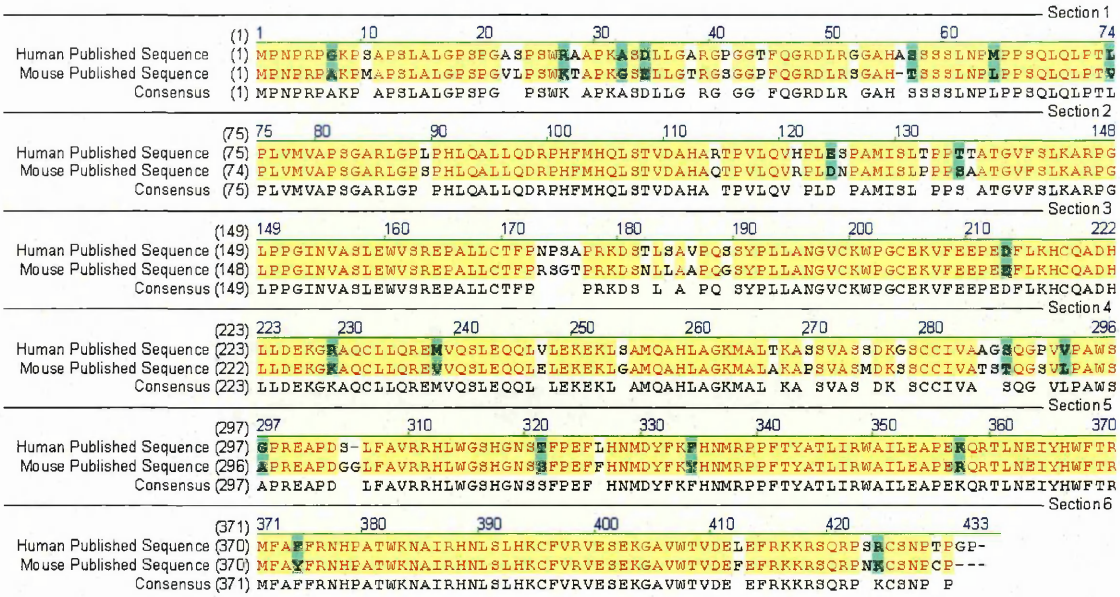


Figure 6.10 Alignment of the published protein sequence for human FOXP3 with the published protein sequence for mouse FOXP3.

Comparison of the two protein sequences confirms that the human form of the protein contains two extra amino acids compared with the mouse protein (431 amino acids versus 429). The proteins show an overall similarity index of 86%. The FKH domain has a similarity index of 94%. Position 173 highlights a region where there is a cluster of 4 amino acids changes. This region maps to exon 4. Red type on a yellow background indicates identical residues. Black type on a blue background indicates similar residues, i.e D and E are both acidic amino acids. Black type on a white background indicates residues with no similarities.

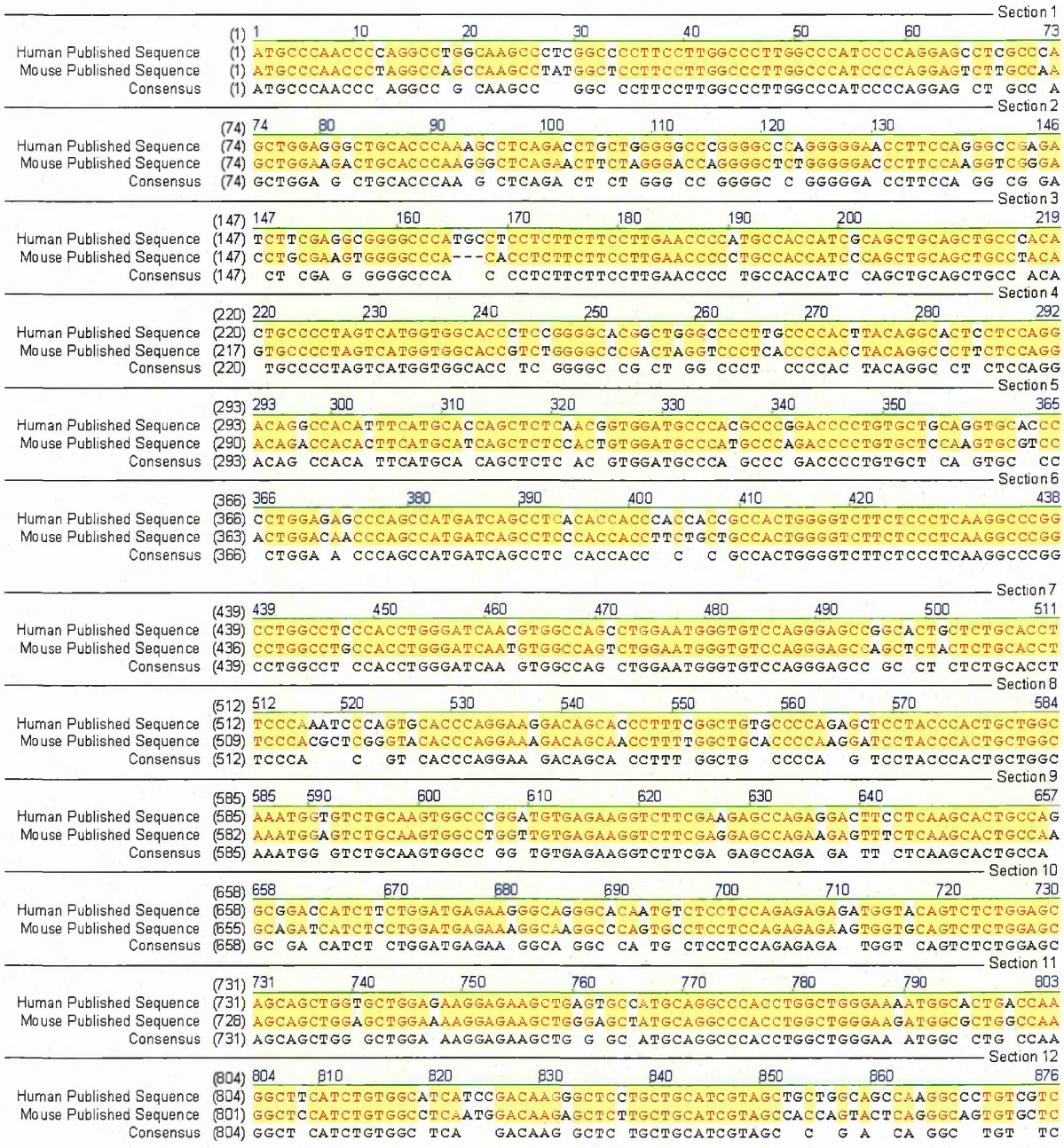


Figure 6.11 continued on next page

Figure 6.11 Alignment of the published nucleotide sequence for human *FOXP3* with the published nucleotide sequence for mouse *Foxp3*.
Comparison of the two nucleotides sequences confirms that the human form of the gene at 1296bp is 6 nucleotides longer that the mouse gene. The genes show 84.4% nucleotide sequence identity.

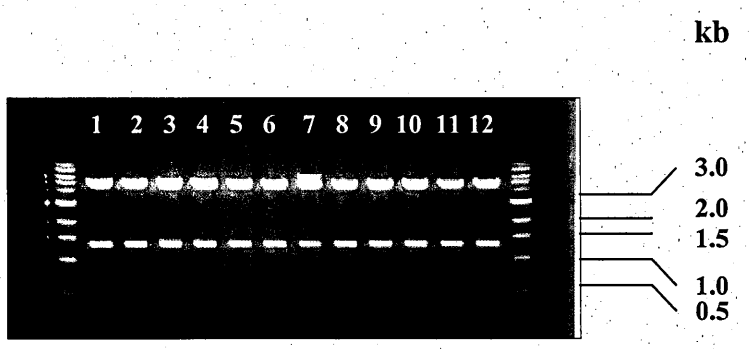


Figure 6.12 Identification of pcDNA3.1+ plasmids containing the full-length mouse *Foxp3* gene.
Small-scale DNA preparations were made of 44 clones. Diagnostic digests were carried out on each clone using the restriction enzymes *Eco*RI and *Not*I. Fragments were separated by gel electrophoresis on a 1% TAE agarose gel. This gel shows the insert fragments for clones 1-12.

Clones 10 and 30 were selected for sequence analysis to confirm that the insert fragment corresponded to the full-length *Foxp3* gene. The sequence alignment data is shown in figure 6.13 and confirms that clones 10 and 30 contained the full-length mouse *Foxp3* gene. Analysis of the 44 clones by restriction enzyme digest and gel electrophoresis, showed that all the clones contained an insert which appeared to be identical in size. On the basis of the sequence data obtained from clones 10 and 30, these data suggest that the 44 clones analysed contained the full length *Foxp3* gene. Whilst not conclusively proving that splice variant forms of the mouse *Foxp3* gene do not exist, these data together with the data from the immunoblot in figure 6.9, strongly suggest that variant forms of the mouse *Foxp3* gene are uncommon.

*Eco*RI

5'- GAGAGAGAATTCGCCACCATGCCCAACCCTAGGCCAGCCAAG - 3'

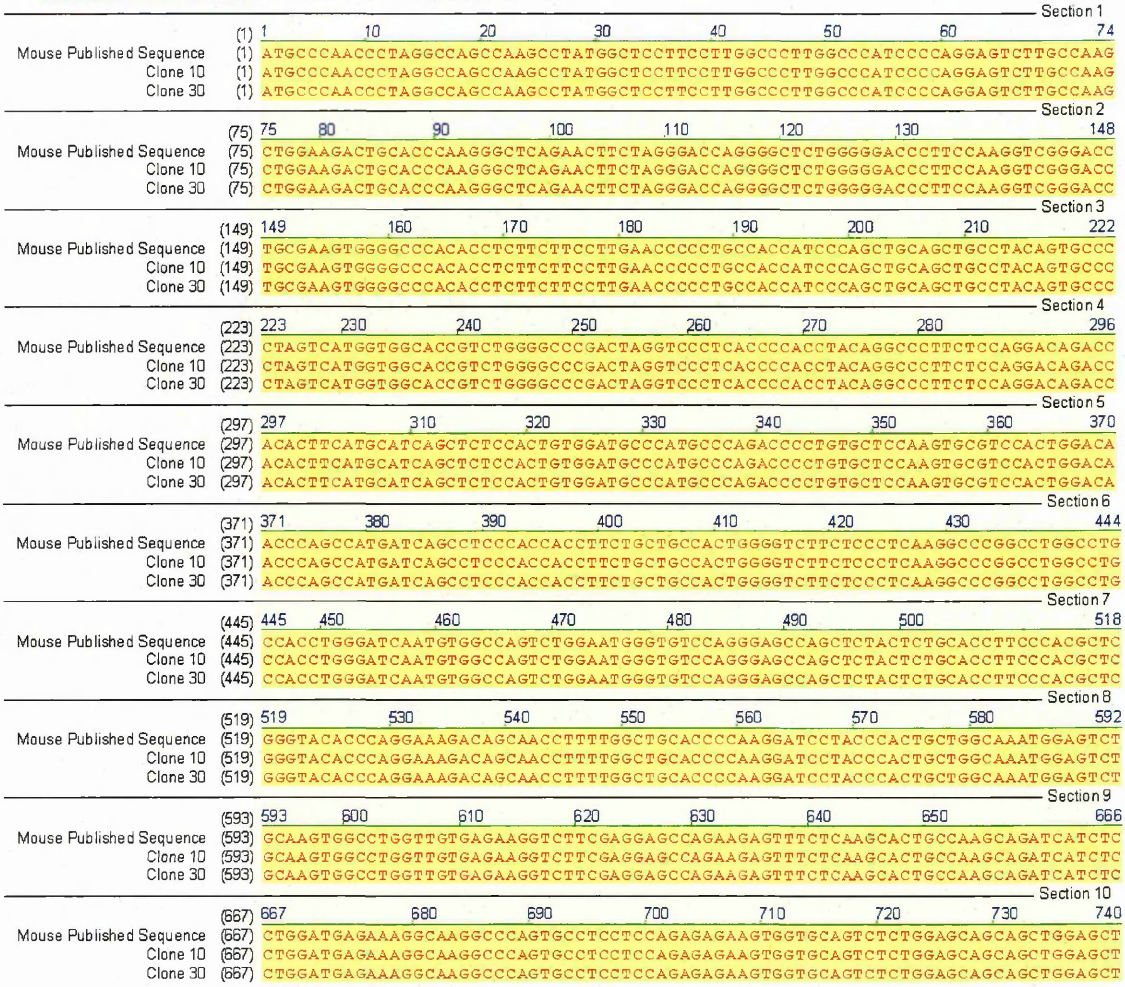


Figure 6.13 continued on next page

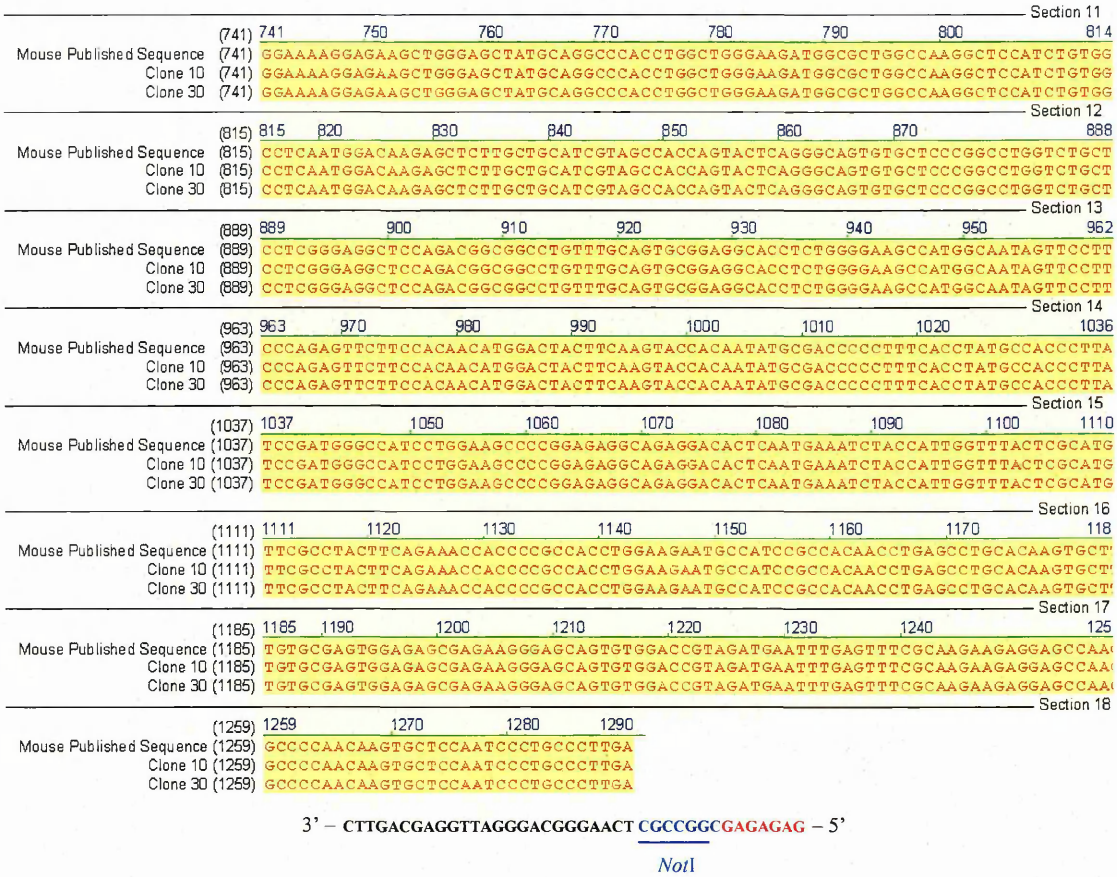


Figure 6.13 Alignment of the published sequence for mouse *Foxp3* with 2 clones obtained from PCR reactions using mouse T cell cDNA.
Sequence analysis of two different clones (10 and 30) produced following amplification of the mouse *Foxp3* gene from activated T cell cDNA derived from the Balb/c mouse. The 5' and 3' cloning primers are shown. The blue residues indicate the enzyme restriction site and the green residues represent a partial Kozak translation initiation site. The red nucleotides protect the 5'-end of the primer.

6.3 Discussion

The functional characterisation of FOXP3 and the FKH mutant in the previous chapter, confirmed that FOXP3 acts as an inhibitor of T cell activation. These data also confirmed that the inhibition observed was critically dependent on the presence of a functional C-terminal FKH domain. During the initial cloning of the human *FOXP3* gene, two novel isoforms of the gene were also identified. Both of the isoforms lacked exon 2 and in addition, clone 10 also lacked exon 7. Exon 2 maps to a proline-rich region of the protein and exon 7 maps to a portion of the leucine-zipper.

It was decided to investigate whether these domains were important for function by determining whether the splice variants were able to inhibit T cell activation induced through the CD28-TCRζ chimeric receptor. Initial transfection studies confirmed that the variant forms of the protein were capable of being expressed in CD4+ T cells, which implied that the missing exons were not affecting the ability of the proteins to be translated. It also appeared that expression levels of the FOXP3 isoforms were higher than both the full-length and the FKH mutant forms of the protein. One explanation for this observation was that the absence of exon 2 somehow improved overall protein stability. Interestingly the variant form of the protein lacking exon 2 also appeared to be expressed in freshly isolated peripheral blood derived CD4+CD25+ regulatory T cells. This strongly suggested a functional role for this isoform.

The data generated from the functional studies showed that both variant forms of the FOXP3 protein significantly inhibited chimeric receptor induced CD4+ T cell

activation, as measured by the release of IL-2. As mentioned in chapter 5, there is a caveat with measuring inhibition of IL-2 production as it could be due to consumption of IL-2. One way to address this would be to include a blocking antibody to CD25. Due to the short nature of the assay, measuring IFNγ production is probably not a suitable alternative. There was also an inhibition of cell surface CD69 expression. These data demonstrated that the lack of exons 2 and 7 did not affect the ability of the FOXP3 protein to inhibit T cell activation. This was surprising since the missing exons encoded protein domains that might have been expected to be key to the function of FOXP3. Indeed gene analysis of an IPEX patient has revealed an in-frame three base-pair deletion (E251) within the leucine zipper (Chatila *et al.*, 2000). In this study, the authors speculated that this deletion prevented dimerisation, thus resulting in the failure of effector function. There has also been a reported mutation within exon 2. However in this case, the mutation was a single base deletion resulting in a frame-shift and a predicted premature termination codon, resulting in a truncated protein lacking the functional FKH domain (Owen *et al.*, 2003).

It is still not clear however if FOXP3 actually needs to dimerise in order to bind to DNA. It had been reported that FKH proteins bind to DNA as monomers (Carlsson and Mahlapuu, 2002). However studies with Foxp1, Foxp2 and Foxp4, three FKH proteins which act as repressors of lung-specific gene transcription (Shu *et al.*, 2001), have shown that these FKH family members do require dimerisation before DNA binding and transcriptional repression can occur. Interestingly this group showed that the zinc-finger domain of these proteins was not essential for transcriptional repression.

Clearly in the studies described in this thesis, the absence of a portion of the leucine-zipper did not prevent the FOXP3 protein from inhibiting T cell activation, suggesting that dimerisation is not required for this function. The absence of a region of the proline-rich region is also not vital for inhibition to occur. As mentioned in the previous chapter, the molecular interactions and targets of FOXP3 are still unknown. It is possible that the signalling pathway that leads to inhibition of T cell activation is distinct from the signalling pathway that generates regulatory activity. It is feasible that the FKH domain is the critical domain for DNA binding and this domain alone is sufficient to inhibit T cell activation. This may occur *via* the active repression of target promoters in for example, proinflammatory genes. Hence each of the three isoforms of the FOXP3 protein investigated, were equally effective at inhibiting chimeric receptor induced CD4+ T cell activation.

Conversely to generate regulatory activity, there might be a requirement for FOXP3 to interact with other proteins to form for example a multiprotein complex. The proline-rich region could be important for bringing different proteins together and the zinc-finger might stablilise this interaction. The leucine-zipper would allow an extra level of post-translational regulation. It is feasible that the FOXP3 protein could form both homo-and heterodimers and these molecules could target the transcription of a different set of genes to the monomeric form of the protein. These genes could be responsible for the generation of regulatory T cell activity.

It is also possible that FOXP3 itself does not need to bind DNA to induce regulatory activity, but by forming a multiprotein complex it allows other as yet unidentified factors to bind to DNA (Schubert *et al.*, 2001). Evidence to support this possibility is

provided by an IPEX patient who does not have a mutation in the coding region of *FOXP3* (Wildin *et al.*, 2001). This patient also exhibited milder symptoms of disease. It is possible that this patient has a mutation within the non-coding sequence of *FOXP3* and this affects transcriptional regulation or RNA splicing. Alternatively this patient could have a mutation in an as yet unidentified protein that forms part of the putative multiprotein complex. This might also explain the milder symptoms, because if FOXP3 does indeed signal through different pathways, the inhibition of T cell activation by the FKH domain would still be functional.

These studies have confirmed that the FKH domain is critical for inhibiting T cell activation. Unfortunately the chimeric receptor system does not allow investigation into the regulatory activity of the FOXP3-transfected cells. However it would be very interesting to determine whether the splice variants also exhibited regulatory activity. As already mentioned, the establishment of a lentiviral-based system allowing continuous expression of the FOXP3 protein, would provide the conditions necessary to investigate this. Another more elegant method for determining whether the variant forms of the FOXP3 protein are capable of also generating regulatory activity, would be to try and use some form of targeted siRNA to repress expression of either exon 2 or exon 7 in naturally occurring CD4+CD25+ regulatory cells. The ability of these cells to regulate could then be investigated. One limitation of this method is the number of CD4+CD25+ regulatory cells recovered from peripheral blood, however there are methods of expanding these cells *in vitro* (Godfrey *et al.*, 2004).

Comparison of the immunoblot of the human and mouse CD4+CD25+ regulatory T cells showed that whilst the human form of the protein appeared to be expressed as a

doublet, the mouse form of the protein was predominantly expressed as a single band, corresponding to full-length FOXP3. Indeed the lower band of the doublet in the human CD4+CD25+ appeared to represent a variant form of the protein lacking exon 2. Scotto *et al.* have confirmed that this isoform of FOXP3, which they refer to as FOXP3α, is expressed in naturally occurring human CD4+CD25+ regulatory T cells (Scotto *et al.*, 2004), although they do not attribute a function to it. Yagi *et al.* have also shown the expression of FOXP3 as a doublet in human CD4+CD25+ regulatory cells and confirmed that the lower band represents a splice isoform lacking exon 2 (Yagi *et al.*, 2004).

Attempts to identify isoforms of the mouse *Foxp3* gene by cloning of the gene and subsequent analysis of clones by diagnostic restriction enzyme digest, proved unsuccessful. Whilst not conclusively proving that isoforms of the mouse FOXP3 protein do not exist, this suggests that if they are expressed, the expression levels are very low and indeed the immunoblot data in figure 6.9 appears to support this. The human and mouse *FOXP3/Foxp3* genes and FOXP3 proteins do however share a high degree of sequence identity (84% at the gene level and 86% at the protein level) and the exon mapping is identical with both full-length proteins consisiting of 11 exons. The appearance of isoforms within the human FOXP3 protein suggests some form of evolutionary pressure may have occurred in the human. This has resulted in the generation of different forms of the protein which may have specific functions.

Currently there is no published data to show whether the two FOXP3 isoforms have regulatory activity. It is possible that both variants do have the ability to generate regulatory activity. However this is thought to be highly unlikely for the isoform

lacking both exons 2 and 7, especially as there is an IPEX patient who exhibits an in-frame mutation within exon 7. Unfortunately the data from the immunoblots was not able to confirm whether the isoform lacking both exons 2 and 7 is naturally expressed in CD4+CD25+ regulatory cells. Nevertheless it was felt that this isoform was unlikely to be a PCR artefact as the missing region mapped to a complete exon. Future studies using monoclonal antibodies to specific exons should be able to address whether this isoform is expressed naturally.

If the isoform lacking exon 2 is naturally expressed in human CD4+CD25+ regulatory cells but not in mouse, this poses the question, what is the functional significance of this isoform? It is possible that this isoform has evolved in humans to limit T cell activation without also playing a role in the signalling pathways required to generate regulatory activity. Evidence is emerging showing that human CD4+CD25- can upregulate FOXP3 following stimulation (Walker *et al.*, 2003a). Whether these cells are generated as an expansion of CD25- T cells expressing low levels of FOXP3, which are already committed to the regulatory T cell lineage, is unclear. Experiments carried out as part of this thesis generation, have also shown the upregulation of FOXP3 in human CD4+CD25- T cells following activation with anti-CD3 and anti-CD28. However the results obtained were inconsistent, with the upregulation of FOXP3 expression not always being obtained, hence the data was not included within this thesis. Nevertheless, when FOXP3 expression was upregulated, it was noted that the lower band of the doublet appeared to be expressed at a higher level than the band corresponding to full-length FOXP3 protein. This suggested that the isoform lacking exon 2 might indeed play a role in controlling T cell activation. It is difficult to ascertain whether this is also true of the immunoblots in the studies of Walker *et al.* It

is also important to note that the activated CD4+CD25- T cells generated in the studies of Walker *et al.* were shown to acquire regulatory activity.

The studies presented in this chapter have confirmed that splice variant forms of the human FOXP3 protein exist. Whether these isoforms each have distinct functions is unclear. Transient expression of the isoforms in human CD4+ T cells showed that both variants significantly inhibited CD28-TCRζ chimeric receptor-induced T cell activation. However the apparent absence of these isoforms in the mouse suggests additional functions for these isoforms which may have evolved over time. Clearly as the signalling pathways involving FOXP3 are elucidated, the significance of these splice variants will become apparent.

Chapter 7

Final Discussion

7.1 Discussion

It is now widely accepted that a population of T cells with regulatory properties do exist within the mammalian immune system. This acceptance has led to a realisation that exploitation of these cells may offer both novel and effective therapies designed to target different clinical diseases. Potentially these cells offer many advantages over conventional therapies. They offer both active and specific regulation and can provide long-term tolerance. Evidence is also emerging to show these cells can be manipulated *ex vivo* to increase cell numbers (Godfrey *et al.*, 2004; Hoffmann *et al.*, 2004; Taylor *et al.*, 2002). However, therapeutic manipulation of these cells also raises several issues. There is the potential for pan immunosuppression if the cells are not antigen specific. This might result in increased risk of infection and/or cancer. The low frequency of these cells is also an issue. As already mentioned, there are protocols for expanding these cells *ex vivo*. However, the potential disadvantage with this treatment currently, is the potential expansion of CD25+ effector T cells.

Clearly, if effective therapies are going to be developed, the biology surrounding these cells needs to be well defined. Currently, the mechanism of suppression by regulatory T cells remains controversial. This is primarily due to differences between *in vitro* and *in vivo* studies, involving the role of cell-cell contact versus soluble cytokines. In an attempt to understand more about the biology of human CD4+CD25+ regulatory T cells, the work presented in this thesis has focussed on characterising human CD4+CD25+ regulatory T cells, both in terms of cell surface marker expression and functionality. The aims were to identify cell surface markers that offered greater specificity than CD25 and also to attempt to identify cell surface

molecules or cytokines that were responsible for mediating suppression. These initial studies were subsequently expanded to investigate the biology of FOXP3 and its role in T cell activation and regulation.

Identification of specific markers which distinguish a population of 'naturally occurring' CD4+ T cells with regulatory activity from conventional CD4+ effector T cells, has provided immunologists with a difficult challenge. If these cells are to be isolated from patients for *ex vivo* manipulation, the purity and specificity of the population will be absolutely paramount. Analysis of cell surface marker expression described in this thesis did not identify any cell surface markers that were truly specific to the CD4+CD25+ regulatory T cell population.

Whilst clearly the analysis of cell surface marker expression described in this thesis was not exhaustive, it does support the concept that a specific cell surface marker for CD4+CD25+ regulatory T cells may not exist. This suggests that T cell-mediated suppression may not be dependent upon one specific molecule. Indeed, the differences in cell surface marker expression between the studies described in this thesis and published studies, highlights the complexity and hetereogeneity of this population of cells. Various groups have carried out detailed microarray analyses and have failed to find a unique marker for these cells that is specific and not expressed on activated CD4+CD25- responder T cells (Gavin *et al.*, 2002; McHugh *et al.*, 2002). It is conceivable therefore that regulatory T cells express markers common to effector T cell and it is the combination of these molecules, or the levels of expression or timing of expression of these shared cell surface markers, which dictates functionality.

Subtle differences in cell surface marker expression may also give rise to subpopulations of regulatory T cells, supporting the idea that regulatory T cells represent a heterogeneous population. As mentioned earlier in this thesis, CD4+CD25+ regulatory T cells expressing different integrins, have the capacity to generate either IL-10 or TGFβ producing regulatory T cells *in vitro* (Stassen *et al.*, 2004). Recent studies have also shown that human CD4+CD25^{hi} regulatory T cells can be subdivided into HLA-DR^{hi} and HLA-DR^{low} expressing cells (C Baecher-Allen, 2005). Whilst both cell populations can suppress responder T cells, the DR^{hi} cells appear to act early in the response to suppress proliferation whilst the DR^{low} act later and also produce IL-10. This level of heterogeneity suggests that there may be specific subsets of naturally occurring regulatory T cell with distinctive modes of suppression that are dispersed within different locations in the body. The local microenvironment may affect the expression of certain cell surface molecules on these cells and this may in part explain some of the differences in mechanism of suppression that have been reported.

The FOXP3 signalling pathway has yet to be elucidated, however forced expression of this molecule is sufficient to convert non-regulatory T cells into regulatory T cells (Hori *et al.*, 2003; Fontenot *et al.*, 2003). How FOXP3 achieves this functional switch is unknown but the FOXP3 signalling pathway does appear to be dependent on TCR signalling (Zahorsky-Reeves and Wilkinson, 2001). One possibility therefore is that encounter of the regulatory T cell with antigen and other cell surface molecules/cytokines, leads to activation of the FOXP3 signalling pathway resulting in the modulation of expression of a number of molecules responsible for generation of a suppressive phenotype.

It is possible that FOXP3 plays an additional role outside of regulatory T cell biology. The studies described in this thesis have shown that FOXP3 appears to play a role in controlling T cell activation. Indeed a recent study has shown that FOXP3 can inhibit IL-2, IL-4 and IFNγ production by human CD4+ T cells following transduction with FOXP3-expressing retrovirus (Bettelli *et al.*, 2005). The studies go on to show that FOXP3 is a specific repressor for NFAT and NF-κB. Whether FOXP3 plays a role in T cell activation during the course of a normal immune response is unclear. The chimeric receptor co-transfection system described in this thesis, the Jurkat T cell system (Schubert *et al.*, 2001) and the recent paper describing overexpression in human CD4+ T cells (Bettelli *et al.*, 2005), have all shown that FOXP3 is capable of inhibiting effector T cell activation. Certainly an additional role for FOXP3 outside of regulatory T cell biology is an attractive one and might in part explain discrepancies in phenotype between CD25-deficient mice and the scurfy mouse.

The identification of splice variant forms of the protein suggests an additional level of complexity surrounding the biology of FOXP3. The functional relevance of these proteins has yet to be determined, although the studies described in this thesis have shown that each splice variant was capable of inhibiting T cell activation as measured by inhibition of IL-2 production. It may be that the isoform lacking exon 2, which is naturally expressed in the human CD4+CD25+ T cell population, plays a role in limiting T cell activation without also playing a role in the signalling pathways required to generate regulatory activity. If the sole function of the isoform lacking exon 2 is to inhibit T cell activation, one might expect freshly isolated CD4+CD25+ thymocytes to only express the full-length version of the gene. Unfortunately there is no published data to confirm or refute this theory. Freshly isolated CD4+CD25+

express both forms of the protein, and this may be because they represent a mixed population of regulatory cells and previously activated cells.

It is also conceivable that CD4+CD25+ regulatory cells isolated from the periphery consist of a mixed population of CD4+CD25+ regulatory T cells derived from the thymus which express full-length FOXP3 and activation-induced CD4+CD25+ regulatory T cells which express the isoform lacking exon 2. Both variant forms of the protein might be capable of inducing regulatory activity and the differences in exon structure might simply be the result of different exon splicing mechanisms within the two cell populations.

The presence of a leucine-zipper and proline-rich regions within the FOXP3 protein suggests that regulatory activity may only occur when the full-length FOXP3 interacts with other proteins forming a multi-protein complex, as discussed in chapter 6. However, it is also conceivable that the splice variants could themselves act as regulators of the full-length protein. During the course of an immune response when an effective CD4+CD25- T cell response is required, the splice variant form of the protein might bind directly to the full-length version of the protein, or to an accessory protein required to form part of the multiprotein complex, and prevent active suppression. Following resolution of the immune response, changes in cytokine levels and/or expression of various cell surface molecules might trigger either increased expression of the full-length FOXP3 protein, or the release of bound full-length protein and/or accessory proteins, resulting in the generation of regulatory activity. Alternatively, the splice variant form of the protein may have evolved to have additional functions distinct from regulatory activity that have yet to be identified.

Clearly the ability of each of the splice variants to generate regulatory activity needs to be investigated. Identification of the frequency of each splice variant form within the mixed CD4+CD25+ T cell population will also aid the investigation into the significance of these variant forms. Analysis of the expression of splice variant forms of FOXP3 in CD4+CD25+ T cells isolated from patients with autoimmune disease may also provide an insight into the functional significance of the different isoforms.

There could be a link between the expression of different isoforms of the FOXP3 protein and disease incidence and symptoms. Clearly in the study of CD4+CD25+ regulatory T cells isolated from patients with RA, the CD4+CD25+ regulatory cells were competent at inhibiting proliferation but not cytokine production (Ehrenstein *et al.*, 2004). This difference may be due to the expression of different isoforms of FOXP3. Determination of the expression profile of FOXP3 in CD4+CD25+ regulatory T cells isolated from patients before and after treatment with anti-TNFα also needs to be investigated. However, this type of frequency analysis is reliant upon the generation of good quality monoclonal antibodies specific to the exons of interest. The use of a bicistronic model system, recently described, may offer an alternative method of investigating the cellular distribution of the splice variants (Wan and Flavell, 2005). This system uses a bicistronic reporter expressing a red fluorescent protein that has been knocked into the *Foxp3* locus, to track expression of FOXP3 in different cell types. Using this system, the group have shown *de novo* FOXP3 expression following anti-CD3 and anti-CD28 antibody-mediated stimulation of CD4+FOXP3- T cells, in the presence of TGFβ.

A second group have also recently reported their results following the use of a GFP-Foxp3 fusion protein-reporter 'knock-in' model (Fontenot *et al.*, 2005). This group showed that FOXP3 expression is highly restricted to αβ T cells and primarily CD4+ T cells, although they do identify a small population of FOXP3+ CD8+ T cells. Interestingly the group demonstrated that a significant proportion of FOXP3+ CD4+ T cells did not express CD25. This was especially true of cells isolated from the spleen and lung, highlighting that CD25 expression does not necessarily correlate with regulatory function. This again might in part explain discrepancies between the phenotype of the CD25-knockout mouse and the scurfy mouse. In contrast to data from other groups and data presented in this thesis, the group argue against a role for FOXP3 in controlling T cell activation and inflammatory cytokine production. They show that OT-II RAG1-/- CD4+ T cells from *Foxp3*- and *Foxp3*+ mice have identical T cell proliferative responses following stimulation with ova peptide. They also claim *de novo* expression of FOXP3 cannot be induced during the course of an immune response.

The future for the area of regulatory T cell biology is both an exciting and challenging one. One of the biggest challenges facing this field is to convert the knowledge surrounding the biology of these cells into a clinical application to treat human diseases including autoimmune disease, cancer and allergy. Studies are being undertaken involving the *ex vivo* expansion of CD4+CD25+ regulatory T cells with the ultimate aim to transfer the cells back into patients (Roncarolo, 2005). However, the potential disadvantage with this treatment, as already alluded to, is the expansion of potential CD25+ effector T cells. One way the group is trying to counter this is by treating the cells with rapamycin that selectively inhibits proliferation of the effector

T cells but not the regulatory T cells (Battaglia *et al.*, 2005). Nevertheless, this method is costly and intensive and even if proved successful, is potentially antigen non-specific. The studies by Fontentot *et al.* have also highlighted the significant proportion of cells that are FOXP3+ but CD25-, supporting the fact that CD25 has limitations as a marker of regulatory T cells. (Fontenot *et al.*, 2005).

Alternatives to *ex vivo* manipulation of regulatory T cells include peptide-based therapies. This type of therapy is much easier to initiate although, it does require knowledge of the antigen responsible for causing disease. In addition, studies using anti-CD25 antibodies to selectively deplete CD4+CD25+ regulatory T cells in cancer may also prove beneficial. One particularly interesting study that emerged during the course of this thesis was the use of chimeric receptors to treat a mouse model of EAE (Mekala and Geiger, 2005). The adoptive transfer of CD4+CD25+ T cells isolated from transgenic mice expressing an MBP-specific chimeric receptor, was effective at inhibiting MBP-peptide induced EAE. These chimeric receptor expressing cells were more effective than non-antigen specific CD4+CD25+ regulatory T cells. However, the really exciting area for therapeutic drug design is likely to reside within the biology of FOXP3.

Determination of the signalling pathways both upstream and downstream of this key molecule should provide a wealth of targets for therapeutic intervention. The studies by Fontentot *et al.* (2005) suggest that FOXP3 is the only truly specific marker of the regulatory T cell lineage, and hence targeting of this molecule should directly affect this T cell subpopulation. Overexpression studies have confirmed its role in regulatory T cell function and additional studies, including those described in this thesis, have

shown a potential additional function for the protein in non-regulatory T cells, through the inhibition of T cell activation. Therefore, direct inhibition of the FOXP3 signalling pathway might be beneficial in cancer treatment. Alternatively molecules that resulted in the upregulation of FOXP3 expression might be beneficial in treatment of autoimmune disease, either through the induction of a T cell population with a regulatory phenotype or simply through the inhibition of activated effector T cells.

The biology of FOXP3 is probably complicated and the identification of splice variant forms of the protein adds an additional layer of complexity. The functionality and frequency of these splice variant forms is yet to be determined. It is feasible that these isoforms have different functions, which might lead to highly specialised therapies that could potentially activate one isoform over the other, dependent upon the desired therapeutic effect.

7.2 Conclusions

The work presented in this thesis has highlighted the complexity of the CD4+CD25+ regulatory T cell population. Whilst CD25 expression is a convenient marker for isolation of regulatory T cells from the resting state, its use is limited following T cell activation. Attempts to identify a more reliable cell surface marker were unsuccessful, although both CD95 and TNFR2 proved to be additional reliable markers for resting CD4+ regulatory T cells. Attempts to investigate the cell surface molecules responsible for suppression of human CD4+CD25- T cells *in vitro* was also unsuccessful and probably reflects the complexity of the mode of action of these cells.

These studies have confirmed the association of FOXP3 expression with the CD4+CD25+ regulatory T cell population. A role for FOXP3 in controlling T cell activation both in CD4+ and CD8+ T cells has also been demonstrated. Novel findings showing that isoforms of human FOXP3 have functionality and can inhibit activation-induced IL-2 production have also been reported. The functional significance of these isoforms requires further investigation and may lead to a better understanding of the biology of FOXP3.

7.3 Future Work

Clearly the biology surrounding FOXP3 and the splice variant forms of the protein warrant further investigation. Future studies include developing a more robust transfection system that allows continous expression of the protein. One possible method is the lentiviral transfection system, as this allows studies to be carried out using both resting and activated T cells. Development of this system would also enable investigation into whether the splice variants are capable of generating suppressor activity, in addition to inhibiting T cell activation.

The generation of stable cell lines expressing each of the variant forms of the protein will allow further investigation into the mechanism of action of FOXP3. Expression could be controlled using an inducible expression system so that the effects of various stimuli could be investigated both pre- and post-FOXP3 expression. Another key area to investigate further is the signalling pathway upstream of FOXP3. Linking the FOXP3 promoter to a reporter gene will enable studies to be undertaken investigating which stimuli drive expression of the FOXP3 gene.

Chapter 8

References

Aandahl EM, Michaelsson J, Moretto W J, Hecht F M and Nixon D F (2004) Human CD4+ CD25+ Regulatory T Cells Control T-Cell Responses to Human Immunodeficiency Virus and Cytomegalovirus Antigens. *J Virol* **78**: pp 2454-2459.

Abel T and Maniatis T (1989) Gene Regulation. Action of Leucine Zippers. *Nature* **341**: pp 24-25.

Akbar AN, Taams L S, Salmon M and Vukmanovic-Stejic M (2003) The Peripheral Generation of CD4+ CD25+ Regulatory T Cells. *Immunology* **109**: pp 319-325.

Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber C B, Blaser K and Akdis C A (2004) Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance Between Allergen-Specific T Regulatory 1 and T Helper 2 Cells. *J Exp Med* **199**: pp 1567-1575.

Alegre ML, Frauwirth K A and Thompson C B (2001) T-Cell Regulation by CD28 and CTLA-4. *Nat Rev Immunol* **1**: pp 220-228.

Anderson MS, Venanzi E S, Klein L, Chen Z, Berzins S P, Turley S J, von Boehmer H, Bronson R, Dierich A, Benoist C and Mathis D (2002) Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. *Science* **298**: pp 1395-1401.

Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, Romagnani P, Maggi E and Romagnani S (2002) Phenotype, Localization, and Mechanism of Suppression of CD4(+)CD25(+) Human Thymocytes. *J Exp Med* **196**: pp 379-387.

Appleman LJ and Boussiotis V A (2003) T Cell Anergy and Costimulation. *Immunol Rev* **192**: pp 161-180.

Arch RH, Gedrich R W and Thompson C B (1998) Tumor Necrosis Factor Receptor-Associated Factors (TRAFs)--a Family of Adapter Proteins That Regulates Life and Death. *Genes Dev* **12**: pp 2821-2830.

Aseffa A, Gumy A, Launois P, MacDonald H R, Louis J A and Tacchini-Cottier F (2002) The Early IL-4 Response to Leishmania Major and the Resulting Th2 Cell Maturation Steering Progressive Disease in BALB/c Mice Are Subject to the Control of Regulatory CD4+CD25+ T Cells. *J Immunol* **169**: pp 3232-3241.

Asseman C, Mauze S, Leach M W, Coffman R L and Powrie F (1999) An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. *J Exp Med* **190**: pp 995-1004.

Baecher-Allan C, Brown J A, Freeman G J and Hafler D A (2001) CD4+CD25high Regulatory Cells in Human Peripheral Blood. *J Immunol* **167**: pp 1245-1253.

Baecher-Allan C (2005) Human CD4+CD25^{high} Treg cells exhibit deficient regulatory function in MS. *Clinical Application of Regulatory T cells Novartis Meeting 7-8 April 2005*.

Battaglia M, Stabilini A and Roncarolo M G (2005) Rapamycin Selectively Expands CD4+CD25+FOXP3+ Regulatory T Cells. *Blood.*

Belkaid Y, Piccirillo C A, Mendez S, Shevach E M and Sacks D L (2002) CD4+CD25+ Regulatory T Cells Control Leishmania Major Persistence and Immunity. *Nature* **420**: pp 502-507.

Bellinghausen I, Klostermann B, Knop J and Saloga J (2003) Human CD4+CD25+ T Cells Derived From the Majority of Atopic Donors Are Able to Suppress TH1 and TH2 Cytokine Production. *J Allergy Clin Immunol* **111**: pp 862-868.

Bennett CL, Christie J, Ramsdell F, Brunkow M E, Ferguson P J, Whitesell L, Kelly T E, Saulsbury F T, Chance P F and Ochs H D (2001) The Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome (IPEX) Is Caused by Mutations of FOXP3. *Nat Genet* **27**: pp 20-21.

Bettelli E, Dastrange M and Oukka M (2005) Foxp3 Interacts With Nuclear Factor of Activated T Cells and NF-{Kappa}B to Repress Cytokine Gene Expression and Effector Functions of T Helper Cells. *Proc Natl Acad Sci U S A* **102**: pp 5138-5143.

Birebent B, Lorho R, Lechartier H, de Guibert S, Alizadeh M, Vu N, Beauplet A, Robillard N and Semana G (2004) Suppressive Properties of Human CD4+CD25+ Regulatory T Cells Are Dependent on CTLA-4 Expression. *Eur J Immunol* **34**: pp 3485-3496.

Blair PJ, Bultman S J, Haas J C, Rouse B T, Wilkinson J E and Godfrey V L (1994) CD4+CD8- T Cells Are the Effector Cells in Disease Pathogenesis in the Scurfy (Sf) Mouse. *J Immunol* **153**: pp 3764-3774.

Brehm MA, Pinto A K, Daniels K A, Schneck J P, Welsh R M and Selin L K (2002) T Cell Immunodominance and Maintenance of Memory Regulated by Unexpectedly Cross-Reactive Pathogens. *Nat Immunol* **3**: pp 627-634.

Bretscher P and Cohn M (1970) A Theory of Self-Nonself Discrimination. *Science* **169**: pp 1042-1049.

Bruder D, Probst-Kepper M, Westendorf A M, Geffers R, Beissert S, Loser K, von Boehmer H, Buer J and Hansen W (2004) Neuropilin-1: a Surface Marker of Regulatory T Cells. *Eur J Immunol* **34**: pp 623-630.

Brunkow ME, Jeffery E W, Hjerrild K A, Paeper B, Clark L B, Yasayko S A, Wilkinson J E, Galas D, Ziegler S F and Ramsdell F (2001) Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse. *Nat Genet* **27**: pp 68-73.

Burkhart C, Liu G Y, Anderton S M, Metzler B and Wraith D C (1999) Peptide-Induced T Cell Regulation of Experimental Autoimmune Encephalomyelitis: a Role for IL-10. *Int Immunol* **11**: pp 1625-1634.

Busch SJ and Sassone-Corsi P (1990) Dimers, Leucine Zippers and DNA-Binding Domains. *Trends Genet* **6**: pp 36-40.

Carlsson P and Mahlapuu M (2002) Forkhead Transcription Factors: Key Players in Development and Metabolism. *Dev Biol* **250**: pp 1-23.

Cederbom L, Hall H and Ivars F (2000) CD4+CD25+ Regulatory T Cells Down-Regulate Co-Stimulatory Molecules on Antigen-Presenting Cells. *Eur J Immunol* **30**: pp 1538-1543.

Chatila TA, Blaeser F, Ho N, Lederman H M, Voulgaropoulos C, Helms C and Bowcock A M (2000) JM2, Encoding a Fork Head-Related Protein, Is Mutated in X-Linked Autoimmunity-Allergic Disregulation Syndrome. *J Clin Invest* **106**: pp R75-R81.

Chen W, Jin W and Wahl S M (1998) Engagement of Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4) Induces Transforming Growth Factor Beta (TGF-Beta) Production by Murine CD4(+) T Cells. *J Exp Med* **188**: pp 1849-1857.

Chen Y, Kuchroo V K, Inobe J, Hafler D A and Weiner H L (1994) Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis. *Science* **265**: pp 1237-1240.

Choi BM, Pae H O, Jeong Y R, Kim Y M and Chung H T (2005) Critical Role of Heme Oxygenase-1 in Foxp3-Mediated Immune Suppression. *Biochem Biophys Res Commun* **327**: pp 1066-1071.

Clark KL, Halay E D, Lai E and Burley S K (1993) Co-Crystal Structure of the HNF-3/Fork Head DNA-Recognition Motif Resembles Histone H5. *Nature* **364**: pp 412-420.

Clark LB, Appleby M W, Brunkow M E, Wilkinson J E, Ziegler S F and Ramsdell F (1999) Cellular and Molecular Characterization of the Scurfy Mouse Mutant. *J Immunol* **162**: pp 2546-2554.

Coffer PJ and Burgering B M (2004) Forkhead-Box Transcription Factors and Their Role in the Immune System. *Nat Rev Immunol* **4**: pp 889-899.

Cong Y, Weaver C T, Lazenby A and Elson C O (2002) Bacterial-Reactive T Regulatory Cells Inhibit Pathogenic Immune Responses to the Enteric Flora. *J Immunol* **169**: pp 6112-6119.

Cosmi L, Liotta F, Angeli R, Mazzinghi B, Santarlasci V, Manetti R, Lasagni L, Vanini V, Romagnani P, Maggi E, Annunziato F and Romagnani S (2004) Th2 Cells Are Less Susceptible Than Th1 Cells to the Suppressive Activity of CD25+ Regulatory Thymocytes Because of Their Responsiveness to Different Cytokines. *Blood* **103**: pp 3117-3121.

Cottrez F, Hurst S D, Coffman R L and Groux H (2000) T Regulatory Cells 1 Inhibit a Th2-Specific Response in Vivo. *J Immunol* **165**: pp 4848-4853.

Crispin JC, Martinez A and Alcocer-Varela J (2003) Quantification of Regulatory T Cells in Patients With Systemic Lupus Erythematosus. *J Autoimmun* **21**: pp 273-276.

Croft M (2003) Co-Stimulatory Members of the TNFR Family: Keys to Effective T-Cell Immunity? *Nat Rev Immunol* **3**: pp 609-620.

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia J R, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis M L, Knutson K L, Chen L and Zou W (2004) Specific Recruitment of Regulatory T Cells in Ovarian Carcinoma Fosters Immune Privilege and Predicts Reduced Survival. *Nat Med* **10**: pp 942-949.

Danke NA, Koelle D M, Yee C, Beheray S and Kwok W W (2004) Autoreactive T Cells in Healthy Individuals. *J Immunol* **172**: pp 5967-5972.

Dao NX and Robinson D S (2004) Fluticasone Propionate Increases CD4CD25 T Regulatory Cell Suppression of Allergen-Stimulated CD4CD25 T Cells by an IL-10-Dependent Mechanism. *J Allergy Clin Immunol* **114**: pp 296-301.

Dhodapkar MV, Steinman R M, Krasovsky J, Munz C and Bhardwaj N (2001) Antigen-Specific Inhibition of Effector T Cell Function in Humans After Injection of Immature Dendritic Cells. *J Exp Med* **193**: pp 233-238.

Dieckmann D, Bruett C H, Ploettner H, Lutz M B and Schuler G (2002) Human CD4(+)CD25(+) Regulatory, Contact-Dependent T Cells Induce Interleukin 10-Producing, Contact-Independent Type 1-Like Regulatory T Cells [Corrected]. *J Exp Med* **196**: pp 247-253.

Dieckmann D, Plottner H, Berchtold S, Berger T and Schuler G (2001) Ex Vivo Isolation and Characterization of CD4(+)CD25(+) T Cells With Regulatory Properties From Human Blood. *J Exp Med* **193**: pp 1303-1310.

Ehrenstein MR, Evans J G, Singh A, Moore S, Warnes G, Isenberg D A and Mauri C (2004) Compromised Function of Regulatory T Cells in Rheumatoid Arthritis and Reversal by Anti-TNFalpha Therapy. *J Exp Med* **200**: pp 277-285.

Fahlen L, Read S, Gorelik L, Hurst S D, Coffman R L, Flavell R A and Powrie F (2005) T Cells That Cannot Respond to TGF-{Beta} Escape Control by CD4+CD25+ Regulatory T Cells. *J Exp Med* **201**: pp 737-746.

Fallarino F, Grohmann U, Hwang K W, Orabona C, Vacca C, Bianchi R, Belladonna M L, Fioretti M C, Alegre M L and Puccetti P (2003) Modulation of Tryptophan Catabolism by Regulatory T Cells. *Nat Immunol* **4**: pp 1206-1212.

Fantini MC, Becker C, Monteleone G, Pallone F, Galle P R and Neurath M F (2004) Cutting Edge: TGF-Beta Induces a Regulatory Phenotype in CD4+. *J Immunol* **172**: pp 5149-5153.

Fehervari Z and Sakaguchi S (2004) CD4+ Tregs and Immune Control. *J Clin Invest* **114**: pp 1209-1217.

Finney HM, Akbar A N and Lawson A D (2004) Activation of Resting Human Primary T Cells With Chimeric Receptors: Costimulation From CD28, Inducible Costimulator, CD134, and CD137 in Series With Signals From the TCRzeta Chain. *J Immunol* **172**: pp 104-113.

Finney HM, Lawson A D, Bebbington C R and Weir A N (1998) Chimeric Receptors Providing Both Primary and Costimulatory Signaling in T Cells From a Single Gene Product. *J Immunol* **161**: pp 2791-2797.

Finney HM (2002) Recombinant Receptor Model for Studying Activation in Human Primary T cells. *PhD Thesis.*

Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R and Salomon B L (2003) Continuous Activation of Autoreactive CD4+ CD25+ Regulatory T Cells in the Steady State. *J Exp Med* **198**: pp 737-746.

Fontenot JD, Gavin M A and Rudensky A Y (2003) Foxp3 Programs the Development and Function of CD4+CD25+ Regulatory T Cells. *Nat Immunol* **4**: pp 330-336.

Fontenot JD, Rasmussen J P, Williams L M, Dooley J L, Farr A G and Rudensky A Y (2005) Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3. *Immunity* **22**: pp 329-341.

Fuss IJ, Boirivant M, Lacy B and Strober W (2002) The Interrelated Roles of TGF-Beta and IL-10 in the Regulation of Experimental Colitis. *J Immunol* **168**: pp 900-908.

Gambineri E, Torgerson T R and Ochs H D (2003) Immune Dysregulation, Polyendocrinopathy, Enteropathy, and X-Linked Inheritance (IPEX), a Syndrome of Systemic Autoimmunity Caused by Mutations of FOXP3, a Critical Regulator of T-Cell Homeostasis. *Curr Opin Rheumatol* **15**: pp 430-435.

Gavin MA, Clarke S R, Negrou E, Gallegos A and Rudensky A (2002) Homeostasis and Anergy of CD4(+)CD25(+) Suppressor T Cells in Vivo. *Nat Immunol* **3**: pp 33-41.

Gershon RK and Kondo K (1970) Cell Interactions in the Induction of Tolerance: the Role of Thymic Lymphocytes. *Immunology* **18**: pp 723-737.

Godfrey VL, Wilkinson J E and Russell L B (1991) X-Linked Lymphoreticular Disease in the Scurfy (Sf) Mutant Mouse. *Am J Pathol* **138**: pp 1379-1387.

Godfrey WR, Ge Y G, Spoden D J, Levine B L, June C H, Blazar B R and Porter S B (2004) In Vitro-Expanded Human CD4(+)CD25(+) T-Regulatory Cells Can Markedly Inhibit Allogeneic Dendritic Cell-Stimulated MLR Cultures. *Blood* **104**: pp 453-461.

Granelli-Piperno A, Golebiowska A, Trumpfheller C, Siegal F P and Steinman R M (2004) HIV-1-Infected Monocyte-Derived Dendritic Cells Do Not Undergo Maturation but Can Elicit IL-10 Production and T Cell Regulation. *Proc Natl Acad Sci U S A* **101**: pp 7669-7674.

Granucci F, Vizzardelli C, Pavelka N, Feau S, Persico M, Virzi E, Rescigno M, Moro G and Ricciardi-Castagnoli P (2001) Inducible IL-2 Production by Dendritic Cells Revealed by Global Gene Expression Analysis. *Nat Immunol* **2**: pp 882-888.

Grohmann U, Fallarino F and Puccetti P (2003) Tolerance, DCs and Tryptophan: Much Ado About IDO. *Trends Immunol* **24**: pp 242-248.

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries J E and Roncarolo M G (1997) A CD4+ T-Cell Subset Inhibits Antigen-Specific T-Cell Responses and Prevents Colitis. *Nature* **389**: pp 737-742.

Gurney AL, Marsters S A, Huang R M, Pitti R M, Mark D T, Baldwin D T, Gray A M, Dowd A D, Brush A D, Heldens A D, Schow A D, Goddard A D, Wood W I, Baker K P, Godowski P J and Ashkenazi A (1999) Identification of a New Member of the Tumor Necrosis Factor Family and Its Receptor, a Human Ortholog of Mouse GITR. *Curr Biol* **9**: pp 215-218.

Higgins SC, Lavelle E C, McCann C, Keogh B, McNeela E, Byrne P, O'Gorman B, Jarnicki A, McGuirk P and Mills K H (2003) Toll-Like Receptor 4-Mediated Innate IL-10 Activates Antigen-Specific Regulatory T Cells and Confers Resistance to Bordetella Pertussis by Inhibiting Inflammatory Pathology. *J Immunol* **171**: pp 3119-3127.

Hoffmann P, Eder R, Kunz-Schughart L A, Andreesen R and Edinger M (2004) Large-Scale in Vitro Expansion of Polyclonal Human CD4(+)CD25high Regulatory T Cells. *Blood* **104**: pp 895-903.

Hori S, Nomura T and Sakaguchi S (2003) Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* **299**: pp 1057-1061.

Huang CT, Workman C J, Flies D, Pan X, Marson A L, Zhou G, Hipkiss E L, Ravi S, Kowalski J, Levitsky H I, Powell J D, Pardoll D M, Drake C G and Vignali D A (2004) Role of LAG-3 in Regulatory T Cells. *Immunity* **21**: pp 503-513.

Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, Newton K, Tschopp J and Strasser A (1999) Activation of Fas by FasL Induces Apoptosis by a Mechanism That Cannot Be Blocked by Bcl-2 or Bcl-x(L). *Proc Natl Acad Sci U S A* **96**: pp 14871-14876.

Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H and Fujii H (2003) Increased Populations of Regulatory T Cells in Peripheral Blood and Tumor-Infiltrating Lymphocytes in Patients With Gastric and Esophageal Cancers. *Clin Cancer Res* **9**: pp 4404-4408.

Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F and Sakaguchi S (1999) Thymus and Autoimmunity: Production of CD25+CD4+ Naturally Anergic and Suppressive T Cells As a Key Function of the Thymus in Maintaining Immunologic Self-Tolerance. *J Immunol* **162**: pp 5317-5326.

Jaeckel E, von Boehmer H and Manns M P (2005) Antigen-Specific FoxP3-Transduced T-Cells Can Control Established Type 1 Diabetes. *Diabetes* **54**: pp 306-310.

Johnson PF and McKnight S L (1989) Eukaryotic Transcriptional Regulatory Proteins. *Annu Rev Biochem* **58**: pp 799-839.

Jonsson H and Peng S L (2005) Forkhead Transcription Factors in Immunology. *Cell Mol Life Sci* **62**: pp 397-409.

Jonuleit H and Schmitt E (2003) The Regulatory T Cell Family: Distinct Subsets and Their Interrelations. *J Immunol* **171**: pp 6323-6327.

Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J and Enk A H (2002) Infectious Tolerance: Human CD25(+) Regulatory T Cells Convey Suppressor Activity to Conventional CD4(+) T Helper Cells. *J Exp Med* **196**: pp 255-260.

Jonuleit H, Schmitt E, Schuler G, Knop J and Enk A H (2000) Induction of Interleukin 10-Producing, Nonproliferating CD4(+) T Cells With Regulatory Properties by Repetitive Stimulation With Allogeneic Immature Human Dendritic Cells. *J Exp Med* **192**: pp 1213-1222.

Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J and Enk A H (2001) Identification and Functional Characterization of Human CD4(+)CD25(+) T Cells With Regulatory Properties Isolated From Peripheral Blood. *J Exp Med* **193**: pp 1285-1294.

Jordan MS, Boesteanu A, Reed A J, Petrone A L, Holenbeck A E, Lerman M A, Naji A and Caton A J (2001) Thymic Selection of CD4+CD25+ Regulatory T Cells Induced by an Agonist Self-Peptide. *Nat Immunol* **2**: pp 301-306.

Kanangat S, Blair P, Reddy R, Daheshia M, Godfrey V, Rouse B T and Wilkinson E (1996) Disease in the Scurfy (Sf) Mouse Is Associated With Overexpression of Cytokine Genes. *Eur J Immunol* **26**: pp 161-165.

Karagiannidis C, Akdis M, Holopainen P, Woolley N J, Hense G, Ruckert B, Mantel P Y, Menz G, Akdis C A, Blaser K and Schmidt-Weber C B (2004) Glucocorticoids Upregulate FOXP3 Expression and Regulatory T Cells in Asthma. *J Allergy Clin Immunol* **114**: pp 1425-1433.

Kay AB (2001) Allergy and Allergic Diseases. First of Two Parts. *N Engl J Med* **344**: pp 30-37.

Kay BK, Williamson M P and Sudol M (2000) The Importance of Being Proline: the Interaction of Proline-Rich Motifs in Signaling Proteins With Their Cognate Domains. *FASEB J* **14**: pp 231-241.

Khattri R, Cox T, Yasayko S A and Ramsdell F (2003) An Essential Role for Scurfin in CD4+CD25+ T Regulatory Cells. *Nat Immunol* **4**: pp 337-342.

Khattri R, Kasprowicz D, Cox T, Mortrud M, Appleby M W, Brunkow M E, Ziegler S F and Ramsdell F (2001) The Amount of Scurfin Protein Determines Peripheral T Cell Number and Responsiveness. *J Immunol* **167**: pp 6312-6320.

Kingsley CI, Karim M, Bushell A R and Wood K J (2002) CD25+CD4+ Regulatory T Cells Prevent Graft Rejection: CTLA-4- and IL-10-Dependent Immunoregulation of Alloresponses. *J Immunol* **168**: pp 1080-1086.

Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, Planta M, McGlaughlin M, Jackson R, Ziegler S F and Fauci A S (2004) CD25(+)CD4(+) Regulatory T Cells From the Peripheral Blood of Asymptomatic HIV-Infected Individuals Regulate CD4(+) and CD8(+) HIV-Specific T Cell Immune Responses in Vitro and Are Associated With Favorable Clinical Markers of Disease Status. *J Exp Med* **200**: pp 331-343.

Kobayashi I, Shiari R, Yamada M, Kawamura N, Okano M, Yara A, Iguchi A, Ishikawa N, Ariga T, Sakiyama Y, Ochs H D and Kobayashi K (2001) Novel Mutations of FOXP3 in Two Japanese Patients With Immune Dysregulation, Polyendocrinopathy, Enteropathy, X Linked Syndrome (IPEX). *J Med Genet* **38**: pp 874-876.

Kreitman RJ (2003) Recombinant Toxins for the Treatment of Cancer. *Curr Opin Mol Ther* **5**: pp 44-51.

Kriegel MA, Lohmann T, Gabler C, Blank N, Kalden J R and Lorenz H M (2004) Defective Suppressor Function of Human CD4+ CD25+ Regulatory T Cells in Autoimmune Polyglandular Syndrome Type II. *J Exp Med* **199**: pp 1285-1291.

Kroczek RA, Mages H W and Hutloff A (2004) Emerging Paradigms of T-Cell Co-Stimulation. *Curr Opin Immunol* **16**: pp 321-327.

Kullberg MC, Jankovic D, Gorelick P L, Caspar P, Letterio J J, Cheever A W and Sher A (2002) Bacteria-Triggered CD4(+) T Regulatory Cells Suppress Helicobacter Hepaticus-Induced Colitis. *J Exp Med* **196**: pp 505-515.

Laity JH, Lee B M and Wright P E (2001) Zinc Finger Proteins: New Insights into Structural and Functional Diversity. *Curr Opin Struct Biol* **11**: pp 39-46.

Landschulz WH, Johnson P F and McKnight S L (1988) The Leucine Zipper: a Hypothetical Structure Common to a New Class of DNA Binding Proteins. *Science* **240**: pp 1759-1764.

Lehmann J, Huehn J, de la R M, Maszyna F, Kretschmer U, Krenn V, Brunner M, Scheffold A and Hamann A (2002) Expression of the Integrin Alpha Ebeta 7 Identifies Unique Subsets of CD25+ As Well As. *Proc Natl Acad Sci U S A* **99**: pp 13031-13036.

Lehmann OJ, Sowden J C, Carlsson P, Jordan T and Bhattacharya S S (2003) Fox's in Development and Disease. *Trends Genet* **19**: pp 339-344.

Levings MK, Sangregorio R and Roncarolo M G (2001) Human CD25(+)CD4(+) T Regulatory Cells Suppress Naive and Memory T Cell Proliferation and Can Be Expanded in Vitro Without Loss of Function. *J Exp Med* **193**: pp 1295-1302.

Liang S, Alard P, Zhao Y, Parnell S, Clark S L and Kosiewicz M M (2005) Conversion of CD4+. *J Exp Med* **201**: pp 127-137.

Ling EM, Smith T, Nguyen X D, Pridgeon C, Dallman M, Arbery J, Carr V A and Robinson D S (2004) Relation of CD4+CD25+ Regulatory T-Cell Suppression of Allergen-Driven T-Cell Activation to Atopic Status and Expression of Allergic Disease. *Lancet* **363**: pp 608-615.

Liu MF, Wang C R, Fung L L and Wu C R (2004) Decreased CD4+CD25+ T Cells in Peripheral Blood of Patients With Systemic Lupus Erythematosus. *Scand J Immunol* **59**: pp 198-202.

Lombardi G, Sidhu S, Batchelor R and Lechler R (1994) Anergic T Cells As Suppressor Cells in Vitro. *Science* **264**: pp 1587-1589.

Malek TR and Bayer A L (2004) Tolerance, Not Immunity, Crucially Depends on IL-2. *Nat Rev Immunol* **4**: pp 665-674.

Malek TR, Porter B O, Codias E K, Scibelli P and Yu A (2000) Normal Lymphoid Homeostasis and Lack of Lethal Autoimmunity in Mice Containing Mature T Cells With Severely Impaired IL-2 Receptors. *J Immunol* **164**: pp 2905-2914.

Malek TR, Yu A, Vincek V, Scibelli P and Kong L (2002) CD4 Regulatory T Cells Prevent Lethal Autoimmunity in IL-2Rbeta-Deficient Mice. Implications for the Nonredundant Function of IL-2. *Immunity* **17**: pp 167-178.

Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders N J and Powrie F (2003) CD4+CD25+ T(R) Cells Suppress Innate Immune Pathology Through Cytokine-Dependent Mechanisms. *J Exp Med* **197**: pp 111-119.

Mason D (1998) A Very High Level of Crossreactivity Is an Essential Feature of the T-Cell Receptor. *Immunol Today* **19**: pp 395-404.

Matzinger P (2002) The Danger Model: a Renewed Sense of Self. *Science* **296**: pp 301-305.

McGuirk P, McCann C and Mills K H (2002) Pathogen-Specific T Regulatory 1 Cells Induced in the Respiratory Tract by a Bacterial Molecule That Stimulates Interleukin 10 Production by Dendritic Cells: a Novel Strategy for Evasion of Protective T Helper Type 1 Responses by Bordetella Pertussis. *J Exp Med* **195**: pp 221-231.

McHugh RS, Whitters M J, Piccirillo C A, Young D A, Shevach E M, Collins M and Byrne M C (2002) CD4(+)CD25(+) Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity* **16**: pp 311-323.

Mekala DJ and Geiger T L (2005) Immunotherapy of Autoimmune Encephalomyelitis With Redirected CD4+CD25+ T Lymphocytes. *Blood* **105**: pp 2090-2092.

Miller DG, Adam M A and Miller A D (1990) Gene Transfer by Retrovirus Vectors Occurs Only in Cells That Are Actively Replicating at the Time of Infection. *Mol Cell Biol* **10**: pp 4239-4242.

Miller JF and Basten A (1996) Mechanisms of Tolerance to Self. *Curr Opin Immunol* **8**: pp 815-821.

Morgan ME, van Bilsen J H, Bakker A M, Heemskerk B, Schilham M W, Hartgers F C, Elferink B G, van der Z L, de Vries R R, Huizinga T W, Ottenhoff T H and Toes R E (2005) Expression of FOXP3 MRNA Is Not Confined to CD4+CD25+ T Regulatory Cells in Humans. *Hum Immunol* **66**: pp 13-20.

Mottet C, Uhlig H H and Powrie F (2003) Cutting Edge: Cure of Colitis by CD4+CD25+ Regulatory T Cells. *J Immunol* **170**: pp 3939-3943.

Muller U, Akdis C A, Fricker M, Akdis M, Blesken T, Bettens F and Blaser K (1998) Successful Immunotherapy With T-Cell Epitope Peptides of Bee Venom Phospholipase A2 Induces Specific T-Cell Anergy in Patients Allergic to Bee Venom. *J Allergy Clin Immunol* **101** : pp 747-754.

Ng WF, Duggan P J, Ponchel F, Matarese G, Lombardi G, Edwards A D, Isaacs J D and Lechler R I (2001) Human CD4(+)CD25(+) Cells: a Naturally Occurring Population of Regulatory T Cells. *Blood* **98**: pp 2736-2744.

Ochs HD, Ziegler S F and Torgerson T R (2005) FOXP3 Acts As a Rheostat of the Immune Response. *Immunol Rev* **203**: pp 156-164.

Oh JW, Seroogy C M, Meyer E H, Akbari O, Berry G, Fathman C G, Dekruyff R H and Umetsu D T (2002) CD4 T-Helper Cells Engineered to Produce IL-10 Prevent Allergen-Induced Airway Hyperreactivity and Inflammation. *J Allergy Clin Immunol* **110**: pp 460-468.

Oldfield WL, Kay A B and Larche M (2001) Allergen-Derived T Cell Peptide-Induced Late Asthmatic Reactions Precede the Induction of Antigen-Specific Hyporesponsiveness in Atopic Allergic Asthmatic Subjects. *J Immunol* **167**: pp 1734-1739.

Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T and Nakayama E (1999) Tumor Rejection by in Vivo Administration of Anti-CD25 (Interleukin-2 Receptor Alpha) Monoclonal Antibody. *Cancer Res* **59**: pp 3128-3133.

Owen CJ, Jennings C E, Imrie H, Lachaux A, Bridges N A, Cheetham T D and Pearce S H (2003) Mutational Analysis of the FOXP3 Gene and Evidence for Genetic Heterogeneity in the Immunodysregulation, Polyendocrinopathy, Enteropathy Syndrome. *J Clin Endocrinol Metab* **88**: pp 6034-6039.

Palmer E (2003) Negative Selection--Clearing Out the Bad Apples From the T-Cell Repertoire. *Nat Rev Immunol* **3**: pp 383-391.

Papiernik M, de Moraes M L, Pontoux C, Vasseur F and Penit C (1998) Regulatory CD4 T Cells: Expression of IL-2R Alpha Chain, Resistance to Clonal Deletion and IL-2 Dependency. *Int Immunol* **10**: pp 371-378.

Pasare C and Medzhitov R (2003) Toll Pathway-Dependent Blockade of CD4+CD25+ T Cell-Mediated Suppression by Dendritic Cells. *Science* **299**: pp 1033-1036.

Paust S, Lu L, McCarty N and Cantor H (2004) Engagement of B7 on Effector T Cells by Regulatory T Cells Prevents Autoimmune Disease. *Proc Natl Acad Sci U S A* **101**: pp 10398-10403.

Piccirillo CA, Letterio J J, Thornton A M, McHugh R S, Mamura M, Mizuhara H and Shevach E M (2002) CD4(+)CD25(+) Regulatory T Cells Can Mediate Suppressor Function in the Absence of Transforming Growth Factor Beta1 Production and Responsiveness. *J Exp Med* **196**: pp 237-246.

Piccirillo CA and Shevach E M (2001) Cutting Edge: Control of CD8+ T Cell Activation by CD4+CD25+ Immunoregulatory Cells. *J Immunol* **167**: pp 1137-1140.

Piccirillo CA and Thornton A M (2004) Cornerstone of Peripheral Tolerance: Naturally Occurring CD4+CD25+ Regulatory T Cells. *Trends Immunol* **25**: pp 374-380.

Pitkanen J and Peterson P (2003) Autoimmune Regulator: From Loss of Function to Autoimmunity. *Genes Immun* **4**: pp 12-21.

Powrie F, Carlino J, Leach M W, Mauze S and Coffman R L (1996) A Critical Role for Transforming Growth Factor-Beta but Not Interleukin 4 in the Suppression of T Helper Type 1-Mediated Colitis by CD45RB(Low) CD4+ T Cells. *J Exp Med* **183**: pp 2669-2674.

Prakken BJ, Samodal R, Le T D, Giannoni F, Yung G P, Scavulli J, Amox D, Roord S, de K, I, Bonnin D, Lanza P, Berry C, Massa M, Billetta R and Albani S (2004) Epitope-Specific Immunotherapy Induces Immune Deviation of Proinflammatory T Cells in Rheumatoid Arthritis. *Proc Natl Acad Sci U S A* **101**: pp 4228-4233.

Putheti P, Pettersson A, Soderstrom M, Link H and Huang Y M (2004) Circulating CD4+CD25+ T Regulatory Cells Are Not Altered in Multiple Sclerosis and Unaffected by Disease-Modulating Drugs. *J Clin Immunol* **24**: pp 155-161.

Ramsdell F (2003) Foxp3 and Natural Regulatory T Cells: Key to a Cell Lineage? *Immunity* **19**: pp 165-168.

Ramsdell F and Ziegler S F (2003) Transcription Factors in Autoimmunity. *Curr Opin Immunol* **15**: pp 718-724.

Read S, Malmstrom V and Powrie F (2000) Cytotoxic T Lymphocyte-Associated Antigen 4 Plays an Essential Role in the Function of CD25(+)CD4(+) Regulatory Cells That Control Intestinal Inflammation. *J Exp Med* **192**: pp 295-302.

Riley JL and June C H (2005) The CD28 Family: a T-Cell Rheostat for Therapeutic Control of T-Cell Activation. *Blood* **105**: pp 13-21.

Robinson DS, Larche M and Durham S R (2004) Tregs and Allergic Disease. *J Clin Invest* **114**: pp 1389-1397.

Roifman CM (2000) Human IL-2 Receptor Alpha Chain Deficiency. *Pediatr Res* **48**: pp 6-11.

Romagnani S (1995) Biology of Human TH1 and TH2 Cells. *J Clin Immunol* **15**: pp 121-129.

Roncarolo MG (2005) *In vitro* and *in vivo* induction of T regulatory 1 cells. *Clinical Application of Regulatory T cells Novartis Meeting 7-8 April 2005.*

Ronchetti S, Nocentini G, Riccardi C and Pandolfi P P (2002) Role of GITR in Activation Response of T Lymphocytes. *Blood* **100**: pp 350-352.

Rouse BT and Suvas S (2004) Regulatory Cells and Infectious Agents: Detentes Cordiale and Contraire. *J Immunol* **173**: pp 2211-2215.

Russell WL, Russell LB and Gower JS (1959) Exceptional Inheritance of a Sex-Linked Gene in the Mouse Explained on the Basis that the X/O Sex-Chromosome Constitution is Female. *PNAS* **45**: pp 554-560.

Sadelain M, Riviere I and Brentjens R (2003) Targeting Tumours With Genetically Enhanced T Lymphocytes. *Nat Rev Cancer* **3**: pp 35-45.

Sadlack B, Merz H, Schorle H, Schimpl A, Feller A C and Horak I (1993) Ulcerative Colitis-Like Disease in Mice With a Disrupted Interleukin-2 Gene. *Cell* **75**: pp 253-261.

Sakaguchi S (2000) Animal Models of Autoimmunity and Their Relevance to Human Diseases. *Curr Opin Immunol* **12**: pp 684-690.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M and Toda M (1995) Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor Alpha-Chains (CD25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases. *J Immunol* **155**: pp 1151-1164.

Salomon B, Lenschow D J, Rhee L, Ashourian N, Singh B, Sharpe A and Bluestone J A (2000) B7/CD28 Costimulation Is Essential for the Homeostasis of the CD4+CD25+ Immunoregulatory T Cells That Control Autoimmune Diabetes. *Immunity* **12**: pp 431-440.

Schubert LA, Jeffery E, Zhang Y, Ramsdell F and Ziegler S F (2001) Scurfin (FOXP3) Acts As a Repressor of Transcription and Regulates T Cell Activation. *J Biol Chem* **276**: pp 37672-37679.

Scotto L, Naiyer A J, Galluzzo S, Rossi P, Manavalan J S, Kim-Schulze S, Fang J, Favera R D, Cortesini R and Suciu-Foca N (2004) Overlap Between Molecular Markers Expressed by Naturally Occurring CD4+CD25+ Regulatory T Cells and Antigen Specific CD4+CD25+ and CD8+CD28- T Suppressor Cells. *Hum Immunol* **65**: pp 1297-1306.

Sharpe AH and Freeman G J (2002) The B7-CD28 Superfamily. *Nat Rev Immunol* **2**: pp 116-126.

Shevach EM (2000) Regulatory T Cells in Autoimmmunity*. *Annu Rev Immunol* **18**: pp 423-449.

Shevach EM (2002) CD4+ CD25+ Suppressor T Cells: More Questions Than Answers. *Nat Rev Immunol* **2**: pp 389-400.

Shimizu J, Yamazaki S and Sakaguchi S (1999) Induction of Tumor Immunity by Removing CD25+CD4+ T Cells: a Common Basis Between Tumor Immunity and Autoimmunity. *J Immunol* **163**: pp 5211-5218.

Shimizu J, Yamazaki S, Takahashi T, Ishida Y and Sakaguchi S (2002) Stimulation of CD25(+)CD4(+) Regulatory T Cells Through GITR Breaks Immunological Self-Tolerance. *Nat Immunol* **3**: pp 135-142.

Shu W, Yang H, Zhang L, Lu M M and Morrisey E E (2001) Characterization of a New Subfamily of Winged-Helix/Forkhead (Fox) Genes That Are Expressed in the Lung and Act As Transcriptional Repressors. *J Biol Chem* **276**: pp 27488-27497.

Smyk-Pearson SK, Bakke A C, Held P K and Wildin R S (2003) Rescue of the Autoimmune Scurfy Mouse by Partial Bone Marrow Transplantation or by Injection With T-Enriched Splenocytes. *Clin Exp Immunol* **133**: pp 193-199.

Sprent J and Kishimoto H (2002) The Thymus and Negative Selection. *Immunol Rev* **185**: pp 126-135.

Stassen M, Fondel S, Bopp T, Richter C, Muller C, Kubach J, Becker C, Knop J, Enk A H, Schmitt S, Schmitt E and Jonuleit H (2004) Human CD25+ Regulatory T Cells: Two Subsets Defined by the Integrins Alpha 4 Beta 7 or Alpha 4 Beta 1 Confer Distinct Suppressive Properties Upon CD4+ T Helper Cells. *Eur J Immunol* **34**: pp 1303-1311.

Stephens GL and Ignatowicz L (2003) Decreasing the Threshold for Thymocyte Activation Biases CD4+ T Cells Toward a Regulatory (CD4+CD25+) Lineage. *Eur J Immunol* **33**: pp 1282-1291.

Stephens LA, Mottet C, Mason D and Powrie F (2001) Human CD4(+)CD25(+) Thymocytes and Peripheral T Cells Have Immune Suppressive Activity in Vitro. *Eur J Immunol* **31**: pp 1247-1254.

Stock P, Akbari O, Berry G, Freeman G J, Dekruyff R H and Umetsu D T (2004) Induction of T Helper Type 1-Like Regulatory Cells That Express Foxp3 and Protect Against Airway Hyper-Reactivity. *Nat Immunol* **5**: pp 1149-1156.

Sugiyama H, Gyulai R, Toichi E, Garaczi E, Shimada S, Stevens S R, McCormick T S and Cooper K D (2005) Dysfunctional Blood and Target Tissue CD4+CD25high Regulatory T Cells in Psoriasis: Mechanism Underlying Unrestrained Pathogenic Effector T Cell Proliferation. *J Immunol* **174**: pp 164-173.

Sundstedt A, O'Neill E J, Nicolson K S and Wraith D C (2003) Role for IL-10 in Suppression Mediated by Peptide-Induced Regulatory T Cells in Vivo. *J Immunol* **170**: pp 1240-1248.

Suri-Payer E, Amar A Z, Thornton A M and Shevach E M (1998) CD4+CD25+ T Cells Inhibit Both the Induction and Effector Function of Autoreactive T Cells and Represent a Unique Lineage of Immunoregulatory Cells. *J Immunol* **160**: pp 1212-1218.

Suzuki H, Kundig T M, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard J J, Ohashi P S, Griesser H and . (1995) Deregulated T Cell Activation and Autoimmunity in Mice Lacking Interleukin-2 Receptor Beta. *Science* **268**: pp 1472-1476.

Taams LS, Smith J, Rustin M H, Salmon M, Poulter L W and Akbar A N (2001) Human Anergic/Suppressive CD4(+)CD25(+) T Cells: a Highly Differentiated and Apoptosis-Prone Population. *Eur J Immunol* **31**: pp 1122-1131.

Taams LS, Vukmanovic-Stejic M, Smith J, Dunne P J, Fletcher J M, Plunkett F J, Ebeling S B, Lombardi G, Rustin M H, Bijlsma J W, Lafeber F P, Salmon M and Akbar A N (2002) Antigen-Specific T Cell Suppression by Human CD4+CD25+ Regulatory T Cells. *Eur J Immunol* **32**: pp 1621-1630.

Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak T W and Sakaguchi S (2000) Immunologic Self-Tolerance Maintained by CD25(+)CD4(+) Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte-Associated Antigen 4. *J Exp Med* **192**: pp 303-310.

Taylor PA, Lees C J and Blazar B R (2002) The Infusion of Ex Vivo Activated and Expanded CD4(+)CD25(+) Immune Regulatory Cells Inhibits Graft-Versus-Host Disease Lethality. *Blood* **99**: pp 3493-3499.

Thornton AM, Donovan E E, Piccirillo C A and Shevach E M (2004) Cutting Edge: IL-2 Is Critically Required for the in Vitro Activation of CD4+CD25+ T Cell Suppressor Function. *J Immunol* **172**: pp 6519-6523.

Thornton AM and Shevach E M (1998) CD4+CD25+ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation in Vitro by Inhibiting Interleukin 2 Production. *J Exp Med* **188**: pp 287-296.

Tiemessen MM, Van Hoffen E, Knulst A C, Van Der Zee J A, Knol E F and Taams L S (2002) CD4 CD25 Regulatory T Cells Are Not Functionally Impaired in Adult Patients With IgE-Mediated Cow's Milk Allergy. *J Allergy Clin Immunol* **110**: pp 934-936.

Tommasini A, Ferrari S, Moratto D, Badolato R, Boniotto M, Pirulli D, Notarangelo L D and Andolina M (2002) X-Chromosome Inactivation Analysis in a Female Carrier of FOXP3 Mutation. *Clin Exp Immunol* **130**: pp 127-130.

Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti J C, Lanzavecchia A and Manz M G (2004) Development of a Human Adaptive Immune System in Cord Blood Cell-Transplanted Mice. *Science* **304**: pp 104-107.

Uraushihara K, Kanai T, Ko K, Totsuka T, Makita S, Iiyama R, Nakamura T and Watanabe M (2003) Regulation of Murine Inflammatory Bowel Disease by CD25+ and. *J Immunol* **171**: pp 708-716.

Verhoef A, Alexander C, Kay A B and Larche M (2005) T Cell Epitope Immunotherapy Induces a CD4(+) T Cell Population With Regulatory Activity. *PLoS Med* **2**: pp e78.

Vieira PL, Christensen J R, Minaee S, O'Neill E J, Barrat F J, Boonstra A, Barthlott T, Stockinger B, Wraith D C and O'Garra A (2004) IL-10-Secreting Regulatory T Cells Do Not Express Foxp3 but Have Comparable Regulatory Function to Naturally Occurring CD4+CD25+ Regulatory T Cells. *J Immunol* **172**: pp 5986-5993.

Viglietta V, Baecher-Allan C, Weiner H L and Hafler D A (2004) Loss of Functional Suppression by CD4+CD25+ Regulatory T Cells in Patients With Multiple Sclerosis. *J Exp Med* **199**: pp 971-979.

von Boehmer H (2005) Mechanisms of Suppression by Suppressor T Cells. *Nat Immunol* **6**: pp 338-344.

von Herrath MG and Harrison L C (2003) Antigen-Induced Regulatory T Cells in Autoimmunity. *Nat Rev Immunol* **3**: pp 223-232.

Walker LS, Chodos A, Eggena M, Dooms H and Abbas A K (2003b) Antigen-Dependent Proliferation of CD4+ CD25+ Regulatory T Cells in Vivo. *J Exp Med* **198**: pp 249-258.

Walker MR, Carson B D, Nepom G T, Ziegler S F and Buckner J H (2005) De Novo Generation of Antigen-Specific CD4+CD25+ Regulatory T Cells From Human CD4+. *Proc Natl Acad Sci U S A* **102**: pp 4103-4108.

Walker R, Kasprowicz D J, Gersuk V H, Benard A, Van Landeghen M, Buckner J H and Ziegler S F (2003a) Induction of FoxP3 and Acquisition of T Regulatory Activity by Stimulated Human CD4+. *J Clin Invest* **112**: pp 1437-1443.

Wan YY and Flavell R A (2005) Identifying Foxp3-Expressing Suppressor T Cells With a Bicistronic Reporter. *Proc Natl Acad Sci U S A* **102**: pp 5126-5131.

Weigel D, Jurgens G, Kuttner F, Seifert E and Jackle H (1989) The Homeotic Gene Fork Head Encodes a Nuclear Protein and Is Expressed in the Terminal Regions of the Drosophila Embryo. *Cell* **57**: pp 645-658.

Weiner HL (2001) Oral Tolerance: Immune Mechanisms and the Generation of Th3-Type TGF-Beta-Secreting Regulatory Cells. *Microbes Infect* **3**: pp 947-954.

Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova J L, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli F D, Byrne G, McEuen M, Proll S, Appleby M and Brunkow M E (2001) X-Linked Neonatal Diabetes Mellitus, Enteropathy and Endocrinopathy Syndrome Is the Human Equivalent of Mouse Scurfy. *Nat Genet* **27**: pp 18-20.

Wildin RS, Smyk-Pearson S and Filipovich A H (2002) Clinical and Molecular Features of the Immunodysregulation, Polyendocrinopathy, Enteropathy, X Linked (IPEX) Syndrome. *J Med Genet* **39**: pp 537-545.

Willerford DM, Chen J, Ferry J A, Davidson L, Ma A and Alt F W (1995) Interleukin-2 Receptor Alpha Chain Regulates the Size and Content of the Peripheral Lymphoid Compartment. *Immunity* **3**: pp 521-530.

Wing K, Lindgren S, Kollberg G, Lundgren A, Harris R A, Rudin A, Lundin S and Suri-Payer E (2003) CD4 T Cell Activation by Myelin Oligodendrocyte Glycoprotein Is Suppressed by Adult but Not Cord Blood CD25+ T Cells. *Eur J Immunol* **33**: pp 579-587.

Woo EY, Yeh H, Chu C S, Schlienger K, Carroll R G, Riley J L, Kaiser L R and June C H (2002) Cutting Edge: Regulatory T Cells From Lung Cancer Patients Directly Inhibit Autologous T Cell Proliferation. *J Immunol* **168**: pp 4272-4276.

Wucherpfennig KW and Strominger J L (1995) Molecular Mimicry in T Cell-Mediated Autoimmunity: Viral Peptides Activate Human T Cell Clones Specific for Myelin Basic Protein. *Cell* **80**: pp 695-705.

Xystrakis E, Dejean A S, Bernard I, Druet P, Liblau R, Gonzalez-Dunia D and Saoudi A (2004) Identification of a Novel Natural Regulatory CD8 T-Cell Subset and Analysis of Its Mechanism of Regulation. *Blood* **104**: pp 3294-3301.

Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S and Sakaguchi S (2004) Crucial Role of FOXP3 in the Development and Function of Human CD25+CD4+ Regulatory T Cells. *Int Immunol* **16**: pp 1643-1656.

Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K and Steinman R M (2003) Direct Expansion of Functional CD25+ CD4+ Regulatory T Cells by Antigen-Processing Dendritic Cells. *J Exp Med* **198**: pp 235-247.

Zahorsky-Reeves JL and Wilkinson J E (2001) The Murine Mutation Scurfy (Sf) Results in an Antigen-Dependent Lymphoproliferative Disease With Altered T Cell Sensitivity. *Eur J Immunol* **31**: pp 196-204.

Zahorsky-Reeves JL and Wilkinson J E (2002) A Transgenic Mouse Strain With Antigen-Specific T Cells (RAG1KO/Sf/OVA) Demonstrates That the Scurfy (Sf) Mutation Causes a Defect in T-Cell Tolerization. *Comp Med* **52**: pp 58-62.

Zinkernagel RM, Ehl S, Aichele P, Oehen S, Kundig T and Hengartner H (1997) Antigen Localisation Regulates Immune Responses in a Dose- and Time-Dependent Fashion: a Geographical View of Immune Reactivity. *Immunol Rev* **156**: pp 199-209.